BACTERIOLOGICAL REVIEWS, Dec. 1975, p. 317-344

Vol. 39, No. 4
Printed in U.S.A.

The Hemolysins of *Staphylococcus aureus*

GORDON M. WISEMAN

Department of Medical Microbiology, University of Manitoba, Winnipeg, R3E OW3 Canada

INTRODUCTION

In 1872, Klebs (113) postulated a relationship between pathogenicity and toxin production in bacteria. Sixteen years later, de Christmas (39) demonstrated toxicity of heated broth cultures of staphylococci recovered from lesions in man. The hemolytic activity of such cultures for rabbit erythrocytes was observed in 1894 by Van de Velde (193) and in 1900 by Kraus and Clairmont (117).

Since that time, bacteriologists have sought to ascribe roles to the many toxins produced by *Staphylococcus aureus*, particularly in relation to pathogenesis and the threat to life. One event more than any focused attention on hemolysin production by these organisms. In 1928, 21 children were inoculated with a diphtheria toxin-antitoxin preparation at Bundaberg, Australia (50). Within 48 h, 12 of the children were dead. A strain of *S. aureus* was isolated from the preparation which apparently stood for some time without refrigeration. The fluid was hemolytic and lethal for rabbits and, in the light of the terminal symptoms shown by the children, it was suggested that a hemolytic toxin was responsible.

This tragedy created increased awareness of the toxic and invasive properties of staphylococci (18, 19), but the nature and mode of action of many of the extracellular products of these organisms is only now becoming better understood.

It is said that *S. aureus* may produce three hemolysins, designated alpha, beta, and delta in the order of their discovery (50, 51). A fourth, gamma hemolysin, was described in 1938 by Smith and Price (178), but their findings were disputed for some years. The lethal and hemolytic effects of cultures on rabbits, established long before the multiplicity of staphylococcal hemolysins was appreciated, were associated with alpha hemolysin. Discovery of the beta hemolysin in 1935 by Glenny and Stevens (71) originated from an investigation of two preparations of alpha hemolysin. They noted that one was neutralized by one-tenth as much antitoxin as the other. Incubation of dilutions of this hemolysin in the presence of sensitive erythrocytes at 37 C, followed by refrigeration at 4 C, caused intensification of hemolysis. Thus the term "hot-cold" has been applied to beta hemolysin. In 1947, Williams and Harper (207) detected delta hemolysin in strains of *S. aureus* grown on sheep blood agar to which alpha and beta antihemolysins were added.

Morgan and Graydon (148) claimed that alpha hemolysin consisted of two distinct lytic substances, "alpha-1" and "alpha-2." They observed that different end points were obtained if two alpha hemolysins which contained no beta hemolysin were neutralized with an antiserum. All culture filtrates contained alpha-1 and about two-thirds had small amounts of alpha-2. It was possible to prepare antisera to both hemolysins.

The gamma hemolysin of Smith and Price was more sensitive to heat than alpha and beta hemolysins, being completely destroyed when held for 30 min at 55 C. Their gamma preparation contained no alpha and only traces of beta hemolysin. Sensitivity of erythrocytes to the hemolysin increased in the order: horse, rat, ox, guinea pig, sheep, man, and rabbit. Slight necrosis was observed when gamma hemolysin was injected into the skin of guinea pigs and rabbits, and it killed the latter but not guinea pigs or mice. Antisera selected to show a high gamma/alpha ratio also had high alpha-2/al-

pha-1 ratios, which suggested to them that gamma hemolysin might be identical with the alpha-2 hemolysin of Morgan and Graydon (148).

Elek and Levy (51) found that the characteristic patterns caused by the hemolysins on blood agar were consistent with the existence of only three hemolysins, alpha, beta, and delta. Thus, seven combinations were possible, all of which they observed. On the basis of these patterns of hemolysis, Elek and Levy decided that alpha-2, gamma, and delta hemolysins were identical, but it now seems clear that their method would not detect gamma hemolysin because it is inhibited by agar (see gamma hemolysin, this review).

In 1951, Marks (137) agreed that alpha-2 and delta hemolysins were identical but argued for the separate existence of gamma hemolysin. He showed that delta hemolysin reacted in the same manner with antisera as alpha-2 hemolysin. Morgan and Graydon (148) had found that most of their culture filtrates contained a small amount of alpha-2 hemolysin which obscured the end point of hemolytic titrations by producing a "tail" of minimal lysis. Alpha-2 hemolysin also acted upon rabbit and sheep erythrocytes. All of these findings were in accordance with those for delta hemolysin. Though Marks was not convinced of the identity of gamma hemolysin with alpha-2 and delta hemolysins, it is noteworthy that the hemolytic spectra of the three were similar. More recently, Jackson (90) suggested that a factor found in his delta hemolysin preparations was identical with gamma hemolysin on the basis of its thermolability and behavior in the presence of mild reducing and oxidizing agents.

PRODUCTION AND PROPERTIES OF THE HEMOLYSINS

Alpha Hemolysin

Production. Probably the best complex media devised are those of Walbum (200–202) and Dolman and Wilson (44). The former contains meat extract, peptone, and $MgSO_4$ buffered at pH 6.8, while Dolman-Wilson medium consists of proteose peptone and a solution of calcium and magnesium salts buffered at pH 7.4. The original formulation of Dolman-Wilson medium required the addition of 0.3% agar. Parker et al. (162) obtained good yields by incubating cultures in 10% CO_2 in air, and Burnet (19) achieved high yields by combining incubation in CO_2 and air with the addition of 0.3% agar to the medium. Variations of Burnet's method have been used by others (120, 125, 167). The effect of CO_2 is not entirely explained by its buffering action since control of pH by alternative means has not been successful. Ganczarski (65) labeled alpha hemolysin with $^{14}CO_2$, and found that CO_2 fixation might play a role in the formation of a key amino acid essential for hemolysin production.

Goode and Baldwin (72) have claimed high yields (2,500 hemolytic units/ml) of the hemolysin in thoroughly aerated Trypticase soy broth supplemented with yeast extract. They found in contrast with many other workers that CO_2 did not enhance titers. Gladstone (66) has shown that oxygen is essential. Enhanced production of hemolysin in the presence of 0.3% agar has been explained on the assumption that it absorbs an unidentified inhibitor (3). Acceptable yields are obtained without agar (9, 32).

Arbuthnott (3) has reviewed the rules which, if adhered to, will ensure satisfactory yields. Alpha hemolysin has also been produced in continuous culture (89).

Limited information is available about nutritional factors which influence production of alpha hemolysin. Several defined media have been investigated, and Gladstone (66) found that amino acids essential in hemolysin production were arginine, glycine, and proline, although some strain variation was observed. Dalen (36–38) confirmed the requirement for arginine and glycine but also noted that serine and histidine increased yields. He observed that histidine caused rapid early production of hemolysin and further suggested that the stimulating effect of CO_2, serine, and glycine was related to their role as precursors of histidine in *S. aureus*. However, stimulation of hemolysin production was not directly correlated with free intracellular histidine.

Duncan and Cho (48) have shown that production of alpha hemolysin is maximal at a glucose concentration of 0.2%. Impairment of nucleic acid and protein biosynthesis resulted in poor yields; the addition of purine and pyrimidine antagonists reduced growth and virulence while tryptophan analogues abolished hemolysin production (124, 180, 190). Reversal of inhibition was achieved with L-tryptophan, indole, or anthranilic acid.

Alpha hemolysin is formed during the logarithmic phase of growth. Duncan and Cho (47) have found that it is released by intact cells as indicated by low levels of deoxyribonucleic acid in the medium at a time of maximal production. The usual finding (3) is that hemolysin production begins in early logarithmic growth and proceeds at a constant rate until the late

log or early stationary phase is reached, although production in these phases has also been observed.

Probably less than 1% of hemolysin is cell associated (47, 142) and this form reaches its maximal level at the onset of the stationary phase. Addition of histidine to cultures induced hemolysin formation intracellularly within 10 min and extracellularly within 15 min (36–38). Coulter and Mukherjee (33) located the hemolysin on the membrane of disrupted staphylococci by means of ferritin-labeled antibody.

Purification. Numerous methods of purification are available for alpha hemolysin and have been reviewed by Arbuthnott (3) and Jeljaszewicz (97). In Arbuthnott's view, one should start with high-titer hemolysin, preferably produced in a defined medium, and concentrate it early in the procedure. This can be conveniently accomplished with methanol or ammonium sulfate. Advantage can be taken of the hemolysin's isoelectric point of 8.6 with the use of preparative electrophoresis and ion-exchange chromatography on diethylaminoethyl-, *O*-(carboxymethyl)cellulose, or diethylaminoethyl Sephadex. The essential features of recent methods of purification are given in Table 1.

Physicochemical characteristics. Purified alpha hemolysin is a protein (3) and claims that it contains carbohydrate (74) have not been substantiated (203). Several amino acid analyses have been performed and are shown in Table 2. The preparation of Fackrell and Wiseman contains a higher concentration of proline, glycine, and alanine than those of other investigators.

Molecular weights of the hemolysin vary from 10^4 to 4.5×10^4 depending upon the method used and other less evident factors (Table 4).

Coulter (32) detected histidine and arginine as the N-terminal amino acids of alpha hemolysin. He suggested that it consisted of two polypeptide chains, but could not separate them by mercaptoethanol and sulfite reduction. The N terminus of alpha hemolysin separated in this way was arginine. Six and Harshman (175) found that the N terminus of hemolysins A and B was alanine, whereas other investigators (215, 217) have identified histidine in this position. Noll (156) has, however, indicated that histidine is an uncommon N terminus. Dalen (37) showed that histidine induces hemolysin production in *S. aureus* Wood 46. The variation in N termini is not surprising since the hemolysin may be nicked in different places by proteolytic enzymes present in crude preparations. This may explain variations in molecular weight noted in different laboratories and in the two forms observed by Six and Harshman (175, 176).

The sedimentation coefficient of alpha hemolysin is in the range 2.8*S* to 3.1*S* (9, 31, 32, 72, 125, 175, 176). Another small fast-moving peak of 12*S* to 16*S* was recorded by Bernheimer and

TABLE 1. *Purification of staphylococcal alpha hemolysin*

Reference	Strain	Procedures[a]	Sp act[b]
Madoff and Weinstein (127)	Wood 46	A, B, C, D	4.2×10^4
Kumar et al. (121)	Wood 46	C, C	?
Kernheimer and Schwartz (9)	Wood 46	A, C, C, E	1.9×10^4
Goshi et al. (74)	?	A, A, D, D	8×10^8
Lominski et al. (125)	Wood 46	A, B, D, A, D	1.2×10^5
Jackson (91)	Wood 46 209-60	A, A, D	?
Robinson and Thatcher (167)	?	A, A, D, C	?
Cooper et al. (31)	Wood 46	A, B, C, D, D	1.2×10^6
Coulter (32)	Wood 46	A, B, C	10^4
Arbuthnott et al. (4)	Wood 46	A, C, C, E, F	2×10^8
Wadstrom (1970a), cited by Jeljaszewicz (97)	Wood 46	B, D, C, A, B	2.2×10^4
Fackrell (53), cited by Wiseman et al. (217)	Wood 46	A, A, B, A, D	1.3×10^5
Goode and Baldwin (72)	Wood 46	A, C, C, G	1.8×10^4
Watanabe and Kato (203)	Wood 46	A, B, C, C, D, C	5.1×10^8

[a] A, Precipitation; B, gel filtration (molecular sieving); C, electrophoresis or electrofocusing; D, ion-exchange chromatography; E, gradient fractionation; F, treatment with heat and urea; G, membrane ultrafiltration.

[b] Hemolytic units per milligram of protein or nitrogen. A hemolytic unit is the highest hemolysin dilution that causes 50% lysis of erythrocytes (216).

TABLE 2. *Amino acid analyses of staphylococcal alpha hemolysin*

Amino acid	Bernheimer and Schwartz (9)[a]	Coulter (32)[b]	Fackrell and Wiseman (56)[b, c]	Six and Harshman (176)[b]	
				A	B
Methionine	10	4	2	6	6
Aspartic acid	44	50	20	40	43
Threonine	23	23	10	22	23
Serine	22	20	10	19	19
Glutamic acid	21	22	16	19	20
Proline	7	10	14	8	9
Glycine	23	24	28	20	24
Alanine	12	14	12	11	11
Valine	12	16	8	13	14
Isoleucine	13	16	6	13	14
Leucine	15	14	8	13	14
Tyrosine	9	10	6	9	10
Phenylalanine	10	10	4	8	8
Lysine	23	26	12	21	23
Histidine	4	4	4	4	4
Arginine	10	8	6	8	8
Ammonia	71	Very high	Very high	?	?

[a] Residues were calculated for a molecular weight of 44,000 as determined by the method of Archibald.
[b] Histidine residues set at 4.
[c] Based on hemolysin of $s_{20,w} = 1.4$.

Schwartz (9) and by Lominski et al. (125). Goode and Baldwin (72) reported a fast-moving component of 10.5*S*. The 12*S* peak seems to be composed of inactive polymerized hemolysin and is separated from the 3*S* fraction by density gradient centrifugation (4). Arbuthnott's (4) observation that 12*S* hemolysin was disaggregated by urea to yield active 3*S* hemolysin supported the contention that it was an inactive form. The 12*S* component was also partially identical with 3*S* material in agar gel diffusion tests. Fackrell and Wiseman (56) obtained 1.4*S* hemolysin when freshly prepared, but several days' standing brought the value to 2.8*S* within the range of most observations. Forlani et al. (57) observed a minor 2*S* component in their 2.8*S* hemolysin. Goode and Baldwin (72) observed a major peak of 3*S* in the ultracentrifuge, a minor peak of 10.5*S*, and a small trailing peak. Dialysis of their preparation against 1 M propionic acid resulted in the appearance of one peak (presumably 3*S*). Hemolysins A and B (175, 176) apparently both give rise to the 12*S* fraction. Bernheimer (8) has observed that as much as 30% of pure 3*S* hemolysin may be in the polymerized 12*S* form, and that the latter has a molecular weight of about 2.4×10^5 to 3.3×10^5.

Electron microscopy studies by Freer et al. (61) showed that negatively stained 12*S* hemolysin consisted of small rings 9 to 10 nm in diameter (outer diameter) which formed part of a hexagonal array of six subunits, each 2.0 to 2.5 nm in diameter, in contrast with amorphous 3*S* hemolysin. It has also been shown (4) that hemolysin heated to 60 C results in a precipitate which can be disaggregated by urea to yield an active preparation. It would seem that the "Arrhenius effect" (in which hemolysin inactivated at 60 C is reactivated by brief heating at 100 C) can be explained on the assumption that 12*S* hemolysin is formed from 3*S* preparations. Thus, further heating at 100 C or treatment with urea disaggregates the insoluble 12*S* component, reforming the active 3*S* fraction.

Bernheimer and Schwartz (9) subjected purified alpha hemolysin to sucrose density gradient centrifugation, finding that three or four electrophoretically distinct but biologically similar peaks were present. Wadstrom (195) found that hemolysin could be separated into components of different isoelectric points (pI). The main component of pI 8.5 comprised about 80% of the hemolytic activity of the strains which produced it. The several fractions were apparently interconvertible. Since all four of Wadstrom's fractions were hemolytically active, they are likely varieties of 3*S* hemolysin, although Bernheimer (8) has stated that 12*S* hemolysin is sometimes hemolytic. McNiven et al. (143) have also found that a main component of hemolysin of pI 8.55 ± 0.12 accounted for 90% of their recovered hemolytic activity, but three additional minor peaks of activity were also noted (Table 3). There are some simi-

rities between their data as shown in the ta-
e. Alpha A and alpha I are the main com-
nents with the same pI, and are reputed to
ow interconversion to alpha B and alpha IV,
spectively. Other investigators (56, 72, 73)
ve also noted pI values in the region of 8.5.
li and Haque (2) have described an alpha and
pha-1 hemolysin on the basis of results ob-
ined with Haque's electrophoretic localiza-
on technique. One (alpha) lysed erythrocytes
rabbits, sheep, and horses whereas the other
lpha-1) lysed only those of the rabbit. Both
ere indistinguishable by electrophoresis and
oved toward the cathode. It is not clear how
ese hemolysins might be related to the com-
nent systems of McNiven and Wadstrom.

A summary of some properties of alpha
molysin is given in Table 4.

Mode of action. A number of workers (24, 102, 111, 140, 216) have shown that alpha hemolysin is bound to erythrocyte membranes. Bernheimer et al. (112) studied the action of hemolysin on erythrocyte membranes by scanning and transmission electron microscopy and found that segments of membrane separated from the cell surface. Most investigators consider the cell membrane to be the primary site of action of the hemolysin. However, numerous studies have been performed in which alpha hemolysin was shown to inhibit ion transport in the toad bladder (164), decrease adenosine 5'-triphosphate levels in tissue cultures (64), and increase oxidation of succinate by Krebs II ascites cells (183). In addition to these findings, Madoff et al. (128) noted that free amino acids were rapidly released from hemolysin-treated Ehrlich ascites cells. Partially purified hemolysin also stimulated adenosine triphosphatase activity in rat liver mitochondria (Novak et al. [158]). In later studies, Novak et al. (159, 160) showed that crude heat-stable alpha hemolysin abolished phosphorylation of adenosine 5'-diphosphate in mitochondria. Partially purified hemolysin also uncoupled oxidative phosphorylation without affecting electron transport. Kadlec and Seferna (100) speculated that the hemolysin may release a loosely bound fraction of membranes in sensitive cells, similar to the action of ouabain. In contrast, Cassidy et al. (25) demonstrated that highly purified alpha hemolysin B had no effect on Ca^{2+}- or (Na^+-K^+)-dependent membrane adenosine

TABLE 3. *Components of the alpha hemolysin of Staphylococcus aureus separated by isoelectric focusing*

McNiven et al. (143)		Wadstrom (195)	
Component	pI	Component	pI
Alpha A[a]	8.55 ± 0.12	Alpha I[b]	8.5
Alpha B	9.15 ± 0.07	Alpha IV	9.2
Alpha C	7.36 ± 0.03	Alpha II	7.0
Alpha D	6.28 ± 0.11	Alpha III	5.0

[a] Alpha A ⇄ Alpha B.
[b] Alpha I ⇄ Alpha IV.

TABLE 4. *Some characteristics of staphylococcal alpha hemolysin*

Reference	Strain	$s_{20,w}$	Mol wt	pI	N termini
Kumar et al. (121)	Wood 46	1.45	1–1.5 × 10^4	—[a]	—
Bernheimer and Schwartz (9)	Wood 46	3.0[b], 12.0[c]	4.4 × 10^4	—	—
Lominski et al. (125)	Wood 46	3.1	—	—	—
Cooper et al. (31)	Wood 46	2.8	—	—	—
Coulter (32)	Wood 46	2.8	2.1 × 10^4	—	Histidine, arginine
Arbuthnott et al. (4)	Wood 46	3.0	—	—	—
Wadstrom (195)	Wood 46, V8, M18	—	—	8.5[b] (alpha I)	—
Wiseman and Caird (215)	Wood 46	—	—	—	Histidine
Forlani et al. (57)	?	2.8	3.3 × 10^4	—	—
McNiven et al. (143)	Wood 46	—	3.6 × 10^4	8.55[a] (alpha A)	—
Six and Harshman (175, 176)	Wood 46	3.0 (A), 3.0 (B)	2.8 × 10^4 (A) 2.6 × 10^4 (B)	7.2 (A), 8.4 (B)	Alanine (A), Alanine (B)
Fackrell and Wiseman (56)	Wood 46	1.4[d]	4.5 × 10^4	8.5	Histidine
Goode and Baldwin (72)	Wood 46	3.0[b] 10.5	—	8.65[b], 5.8	—
Goode and Baldwin (73)	57, 2079, 3558, 3565	3.0[b] 10.5	—	8.65 ± 0.15	—
Watanabe and Kato (203)	Wood 46		3.6 × 10^4	7.98 ± 0.05	—

[a] Not reported.
[b] Major component.
[c] Inactive aggregate.
[d] Unstable; after standing several days, $s_{20,w}$ was 2.8.

triphosphatase, proton translocation in mitochondria, or the integrity of membranes of *Mycoplasma*. As indicated by Cassidy, these observations require careful interpretation since hemolysin of undefined purity was often employed. Rahal (163) also failed to show interaction of hemolysin with mitochondrial membranes.

The action of alpha hemolysin on artificial membranes has also been studied by several investigators. Weissman et al. (204) showed that hemolysin released ions from artificial lipid spherules and found that the reaction was inhibited by alpha antihemolysin. They suggested that the hemolysin through hydrophobic moieties located within its molecule interacted with phospholipids in sensitive membranes. This view was also put forward by Arbuthnott (3). Freer et al. (61) showed that the hemolysin disrupted spherules with the formation of ring structures which resembled 12S hemolysin under the electron microscope. Apparently, interaction between hemolysin and phospholipid took place, which resulted in polymerization of 3S to 12S hemolysin. Arbuthnott et al. (5) have stated that certain lipids induce polymerization of 3S hemolysin to the 12S form. Polymerization was induced by the following lipids in order of their decreasing sensitivity: diglyceride, lecithin, cholesterol, and lysolecithin. However, a mixture of lecithin, cholesterol, and dicetyl phosphate (70:10:20 molar ratio) was more successful in promoting polymerization than any individual lipid. They concluded that the polar group of lecithin is not required for polymerization and that the alpha hemolysin can interact hydrophobically with lipids as concluded earlier by others (61, 204).

Because alpha hemolysin disrupts artificial and natural membranes, Buckelew and Colacicco (17) studied its behavior at air-water interfaces. They found that the hemolysin spread readily as a thick film on aqueous media and that penetration of hemolysin into lipid monolayers was related to their structure. Rates of penetration were greatest with cholesterol and least with gangliosides. Other lipids such as sphingomyelin, phosphatidylinositol, and lecithin occupied an intermediate position. They argued that surface activity of alpha hemolysin may play an important role in its action upon membranes. In the light of these results, it is curious that Weissman et al. (204) found that alpha hemolysin released the same amount of anions from spherules whether or not cholesterol was present.

Inhibition of alpha hemolysin activity by ganglioside was also noted by Colacicco and Buckelew (29) and some years earlier by Doery and North (41). The latter authors observed that ganglioside inactivated the lethal effects of hemolysin in mice. Wiseman (unpublished observations) studied the interaction of hemolysin and ganglioside by difference spectral techniques in the ultraviolet region of the spectrum. He noted that the absorption peak of hemolysin underwent a small shift toward the red end of the spectrum, but that a similar shift was observed if hemolysin was incubated with albumin or casein. It thus appears that reaction of alpha hemolysin with ganglioside is nonspecific, but this does not rule out the possibility that hemolysin-ganglioside interaction is in some way responsible for hemolysis.

Harvie (84) associated alpha hemolysin with cholesterol esterase activity, since her hemolysin preparation deesterified cholesteryl esters in lipoproteins. She made no attempt to correlate this activity with the hemolytic spectrum of the hemolysin.

Great importance has been attached to the presence of ringlike structures (Fig. 1) on hemolysin-treated artificial and natural membranes negatively stained and viewed under the electron microscope (4, 5, 11, 61, 62). These rings are reported as having diameters of 8.7 (165), 10 (4), and 9 to 10 nm (61). According to Bernheimer (8), it seems uncertain whether the rings are formed before or after lysis takes place. The view that rings are inactive (polymerized) 12S hemolysin is based on morphological evidence and on the observation that incubation of ring-containing membranes with 8 M urea releases hemolytic activity. Arbuthnott et al. (5) have also found that sodium dodecyl sulfate disc gel electrophoresis of active 3S hemolysin yielded a slow-moving component (12S) which contained the ring structures. The rings are associated only with mammalian cell membranes and are not found on bacterial membranes exposed to hemolysin (11). Perhaps the latter are deficient in hemolysin activator, or it may be that hemolysin interacts with lecithin, common in mammalian but rare in bacterial membranes. The ring phenomenon is also seen in erythrocyte membranes undergoing immune hemolysis (172). According to Seeman (172), there is a close association between globules of protein in the membranes and the rings. Thus the formation of rings in membranes incubated with alpha hemolysin may merely be a consequence of hemolysis induced by a variety of agents.

Bernheimer et al. (11) have also shown that freeze-etching of hemolysin-treated artificial

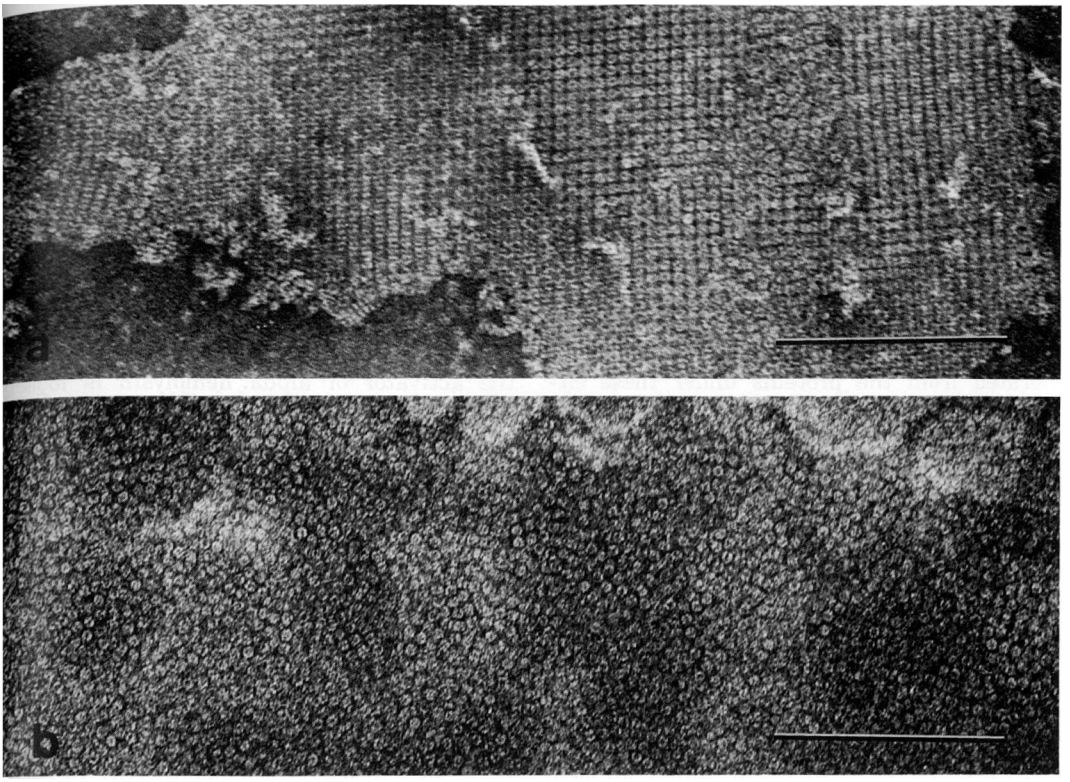

FIG. 1. *(a) Rectangularly ordered rings on a portion of a rabbit blood platelet fragment treated with a mixture of 90% α-toxin and 10% δ-toxin and negatively stained with ammonium molybdate. Bar represents 0.2 nm. (b) Randomly disposed rings on portion of rabbit blood platelet membrane treated with a mixture of 99% α-toxin and 1% δ-toxin and negatively stained with ammonium molybdate. Bar represents 0.2 nm (× 150,000). Figure is reproduced from Bernheimer's article (8).*

and natural membranes revealed a rectangular or randomly distributed array of rings. Random orientation of rings was associated only with the effects of alpha hemolysin, whereas rectangular patterns were observed when alpha and delta hemolysins were added together. These observations are different from those of Freer et al. (62), who found that freeze-fractured erythrocyte membranes incubated with alpha hemolysin were vesiculated and fragmented. Ring structures were not observed in these preparations. High magnification micrographs of freeze-etched membranes showed smooth depressions and plaque formation but no rings. Similar depressions (pits) were also observed in freeze-etched erythrocyte membranes incubated with saponin (172). It is difficult to reconcile these findings with those of Bernheimer, but small differences in methodology and in hemolysin preparations might grossly affect the appearance of the membranes. Relevant perhaps is Bernheimer's (11) statement that his alpha hemolysin contained 1 part delta hemolysin in 400 parts of alpha hemolysin. Thus, small quantities of contaminating delta hemolysin in otherwise homogeneous alpha hemolysin could account for the appearance of randomly ordered rings in contrast with the rectangular arrays seen with larger proportions of delta to alpha hemolysin. The alpha hemolysin of Freer et al. (62) was claimed to be free from other extracellular products.

Cassidy et al. (25) have called into question the usefulness of artificial membranes as models for the investigation of hemolysin action. They found that the action of alpha hemolysin on spherules composed of rabbit erythrocyte phospholipid was not selective. Spherules which contained phospholipid from human erythrocytes were equally sensitive to the hemolysin. This finding is not in agreement with the observation that human erythrocytes are many times more resistant to alpha hemolysin than rabbit erythrocytes.

Another view of the mode of action of alpha hemolysin has recently been put forward by

Wiseman and Caird (215, 216) and Wiseman et al. (217). They found that, although hemolysin released no acid-soluble phosphorus from rabbit erythrocyte membranes, nitrogen appeared in the supernatant fluid and increased proportionally with respect to time (215). The same phenomenon was observed with lipid-free rabbit erythrocyte membrane protein. In particular, release of nitrogen from erythrocytes by hemolysin was directly correlated with their hemolytic sensitivity. Hemolysin had no effect upon hemoglobin and several other proteins tested, unless an erythrocyte membrane preparation was also added. The amount of nitrogen liberated from the proteins under these circumstances was greater than that released by the membranes alone. Morrison and Neurath (149) and Moore et al. (147) have studied the proteolytic action of erythrocyte membranes, and it appeared to us that the enhanced release of nitrogen from test proteins by hemolysin–"ghost" mixtures might be due to activation of alpha hemolysin by membrane proteinases. The proteolytic activity of erythrocyte ghosts was directly correlated with their hemolytic sensitivity. Analysis of erythrocyte membranes treated with hemolysin is shown in Table 5 and indicates that the protein content is reduced.

Further evidence in support of these conclusions has been obtained by Wiseman et al. (217), who showed that alpha hemolysin is activated by trypsin and that it will then hydrolyze tosyl arginine methylester. The hemolysin was activated by trypsin coupled with carboxymethylcellulose in the presence of *N,N'*-dicyclohexylcarbodiimide. The K_m of the hemolysin was different from that of the trypsin although both exhibited the same V_{max} with tosyl arginine methylester as substrate.

TABLE 5. *Analysis of rabbit erythrocyte membranes treated with staphylococcal alpha hemolysin*[a]

Assay	mg of packed cells/ml[b]	
	Untreated membranes	Membranes treated with alpha hemolysin
Phospholipid	1.19	1.20
Total lipid	1.45	1.50
Cholesterol	0.33	0.34
Total carbohydrate	0.26	0.27
Hexosamine	0.14	0.14
Sialic acid	0.12	0.12
Pentose (as ribose)	0.07	0.06
Nitrogen	0.50	0.39
Nitrogen as protein[c]	3.10	2.42
Protein[d]	1.84	1.66

[a] Wiseman (unpublished observations).
[b] Mean of three determinations.
[c] Nitrogen concentration multiplied by 6.25.
[d] Determined by the Lowry reaction.

Binding of ^{125}I-hemolysin to erythrocytes is correlated with their hemolytic sensitivity (24) and confirms earlier work (216). Cassidy and Harshman (24) observed that, after partial liberation of ^{125}I-hemolysin from erythrocytes, a final phase was reached in which a portion of it remained bound to the membrane. These findings are to some extent in keeping with the observation of Wiseman and Caird (216) that the activator of alpha hemolysin is loosely bound to rabbit erythrocyte membranes. They found that a portion remained on the membrane and some appeared in the supernatant complexed with activator. Apart from this it is not possible to say how accurately Cassidy's findings reflect the system proposed by Wiseman and Caird. Freer et al. (62) could not confirm evidence of a proteolytic mechanism in the mode of action of alpha hemolysin. They found that rabbit erythocyte stromata treated with hemolysin showed (i) no alteration in polypeptide patterns as assessed by disc gel electrophoresis which might be expected if a proteinase was involved; (ii) no reduction in sedimentable protein; (iii) a freeze-etching pattern under the electron microscope unlike that obtained with known proteolytic enzymes. A fourth observation was that the proteinase inhibitor phenylmethane sulfonylfluoride failed to inhibit hemolysis. It should be pointed out, however, that Freer et al. made no attempt to demonstrate the presence of activator in their membrane preparations. Ghost preparations can vary in properties with respect to the method of preparation (Dodge et al. [40]), and they may have lost or destroyed it. There is also no reason to suppose that the freeze-etching pattern obtained in hemolysin-treated erythrocyte membranes will resemble that of all other proteolytic enzymes. For example, Speth et al. (182) did not observe a common freezing-etching pattern in membranes treated with phospholipases.

The problem at hand is the reconciliation of two views of the interaction of alpha hemolysin with its substrate in the erythrocyte membrane. One is that the surface activity of the hemolysin and its interaction with lipids accounts for its biological activity. The other view argues that the erythrocyte membrane activates the hemolysin by a proteolytic mechanism, and the activated material can react with membrane proteins producing lysis. As we have stated,

Cassidy et al. (25) found that spherules composed of lipid extracted from human and rabbit erythrocytes were equally sensitive to alpha hemolysin in contrast with the known differences in hemolytic sensitivities of these erythrocyte species. Moore et al. (147) in their study of erythrocyte proteinases found that activity was associated with membrane lipoprotein. Perhaps the interaction of alpha hemolysin with membrane lipoproteins might anchor it in a particular conformation which is susceptible to the action of proteinase activator.

Toxic properties of alpha hemolysin. Various definitions of a toxin have been put forward, but Bonventre (15) defined a toxin as a high-molecular-weight protein of microbiological origin which is antigenic and causes disruption of normal physiological processes in a sensitive animal. Although this definition has been challenged (194), alpha hemolysin fulfills these criteria and may be called a toxin.

Both cold- and warm-blooded animals are sensitive to the hemolysin and the severity of its effects is dose dependent. Fackrell and Wiseman (56) determined that the mean lethal dose (LD_{50}) in mice injected intraperitoneally was 0.68 ± 0.19 μg or 27 to 34 μg/kg of body weight, in fair agreement with the value obtained by Arbuthnott (3). Lominski et al. (125) obtained minimum lethal dose values of 50 μg/kg in mice and 1.3 μg/kg in rabbits injected by the intravenous route. LD_{50} data obtained by others for mice are 600 μg/kg (74) and about 50 μg/kg intravenously (203). Watanabe and Kato (203) found that the minimum dermonecrotic dose in rabbit skin was 0.03 μg. Rabbits injected intravenously with a minimal lethal dose die after a few days. The major pathological findings are kidney necrosis frequently accompanied by flaccid paralysis of the hind legs. Larger intravenous doses may cause death in a few minutes, unsteadiness, or respiratory difficulty and intermittent muscular spasms. However, at death the limbs are flaccid and no histological changes appear evident. Intravascular hemolysis has been noted in animals given large doses. Subcutaneous administration of small doses results in what appears to be hemolysis under the skin in the surrounding area, which progresses to severe necrosis, sloughing of tissue, and heavy scab formation after several days.

Arbuthnott (3), in summarizing the main findings, has observed that the hemolysin acts on the peripheral circulation, heart, and central nervous system. In perfused heart muscle from the rabbit, chicken, and cat, alpha hemolysin caused constriction of the coronary arteries and systolic arrest (205, 206). Nelis (153) also suggested direct action on the respiratory center. Samanek and Zajic (170) have reported a decline in cardiac output in animals that have received hemolysin. The latter group noted changes in blood pressure which they suggested were caused by an effect of the hemolysin on smooth muscle of the blood vessels. In keeping with this is the observation of Thal and Egner (187, 188) that hemolysin causes vasospasm of the small blood vessels. Jeljaszewicz et al. (99) have studied the distribution of ^{131}I-alpha hemolysin in rabbits, finding that intravenous injection distributed toxin in nearly all organs but in increased amount in kidney and lungs. It was also detected in brain tissue. Cassidy et al. (25) showed that alpha hemolysin in 1-μg/ml amounts induced spastic paralysis of the smooth muscle of guinea pig ileum, a phenomenon which was unresponsive to K^+ or Ca^{2+} ions or isoproteranol. Wurzel et al. (218) made similar observations. This range of findings indicates, as observed by Arbuthnott (3), that there is no unified concept of the action of alpha hemolysin in the animal body. The proteolytic mechanism of action might in part explain its effect on animals by assuming that it selectively destroys those cells in vivo which have the highest membrane content of activating proteinase. This hypothesis, however, is unable to account for rapid death in sensitive animals given large doses.

Numerous studies of the toxic effect of alpha hemolysin on cultured cells have been reported (97). Ehrlich ascites cells have been used (128), as well as KB cells (115, 116), chicken embryo cells (64, 155), rabbit kidney (64, 161, 189), calf, human, and monkey kidney cells (80), and human diploid fibroblasts and HeLa cells (189).

Alpha hemolysin induces blast transformation in rabbit lymphocyte cultures (35), and its effect on platelets (10, 136, 157), leukocytes, and macrophages (67, 134) has also been studied. The action of hemolysin has been investigated on almost every type of cell and the effects described are legion. Probably most of the findings could be explained on the basis of the primary interaction of hemolysin with cell membranes. Discussion of the cytotoxicity of alpha hemolysin is also found in the reviews of Jeljaszewicz (97) and Arbuthnott (3).

Beta Hemolysin

Production. The production of beta hemolysin on solid and in liquid media has been reviewed by Wiseman (211). As with alpha hemolysin, incubation of cultures in a mixture of CO_2 and air or oxygen gives good yields,

concentrations in the range of 10 to 25% having been found satisfactory by most investigators (26, 27, 43, 75, 90, 168). Maheswaran et al. (133) found an atmosphere of 50% CO_2 in oxygen adequate, but Haque and Baldwin (82) noted no difference in titers over a range of 20 to 80% CO_2 in air or 20% CO_2 in oxygen. Wiseman (211) reported that production of beta hemolysin was decreased in mixtures of CO_2 in oxygen in contrast with CO_2 in air. Wadstrom and Mollby (196) obtained sufficient yields in liquid media adequately aerated in the absence, presumably, of CO_2. Like alpha hemolysin, the role of CO_2 in enhancement of beta hemolysin titers is not specifically understood, but effects upon growth do not appear to be responsible (211).

Limited information is available regarding nutritional factors which affect production of beta hemolysin (211). An investigation of the amino acid requirements of *S. aureus* R-1 was made and it was found that arginine, proline, and glycine were an absolute requirement, as were cystine and methionine together but not separately (Table 6). Thiamine and nicotinamide were indispensable. There was no clear distinction between amino acid requirements for growth and hemolysin production. Chesbro et al. (27) supplemented their completely dialyzable medium with 0.5% arginine and achieved improved yields.

Of interest is the recent paper by Sharma and Haque (173) in which the 681C strain of *S. aureus* was grown in a liquid synthetic medium. Beta hemolysin production in this strain required proline, glutamine, and cystine. Aeration by shaking was inhibitory, but incubation of the cultures under 15% CO_2 tension increased yields. The 681C strain has somewhat different requirements compared to the R-1 strain. Sharma and Haque (174) have noted that disturbances in tryptophan metabolism affect beta hemolysin formation, as judged by its inhibition in the presence of tryptophan analogues such as 5-fluorotryptophan. This finding is similar to that observed for alpha hemolysin.

Beta hemolysin is produced at a maximal rate early in the logarithmic phase of growth (211).

Purification. Procedures developed for beta hemolysin have been extensively reviewed (97, 211). A summary of the main features is found in Table 7.

Physicochemical characteristics. Numerous studies have shown that the hemolysin is a protein (83, 92, 133, 208). Molecular weights range from 1.1×10^4 to 5.9×10^4 (26) to values of 1.6×10^4 to 3.8×10^4, depending upon the strain (see Table 9). The amino acid composition is given in Table 8. There are some differences between Fackrell's data and those of Bernheimer et al. as shown in Table 8, but the cause of the variation is not clear.

TABLE 6. *Nutritional requirements of Staphylococcus aureus R-1 in a defined solid medium*[a]

Omissions from complete medium	Growth (OD_{650})[b]	Hemolytic titer of beta hemolysin (HU/ml)[c]
Alanine	0.09	640
Leucine	0.07	640
Valine	0.03	160
Glycine	0.01	40
Hydroxyproline	0.08	640
Tryptophan	0.08	320
Histidine	0.08	320
Proline	0.01	<20
Aspartic acid	0.08	320
Glutamic acid	0.08	320
Cystine	0.06	160
Methionine	0.08	640
Cystine, methionine	0.02	<20
Lysine	0.09	640
Arginine	0.02	<20
Thiamine	0.03	<20
Nicotinamide	0.04	20
Complete medium[d]	0.08	640

[a] Modified from Wiseman (211).

[b] OD_{650}, Optical density at 650 nm; cells were harvested and diluted 1:20 before optimal density measurements were made.

[c] Sheep erythrocytes; HU, hemolytic units (see 216).

[d] Based on Gladstone (66).

Several investigators have separated a major (cationic) and a minor (anionic) component from preparations of beta hemolysin (83, 133, 145). Mollby and Wadstrom (145) found that 95% of their crude hemolysin subjected to isoelectric focusing was in the cationic form of pI = 9.5 ± 0.1. About 5% of the crude preparation was in the anionic form (pI = 3 to 4). They also showed that occurrence of the anionic hemolysin was probably due to aggregation (presumably of some of the cationic form) and that passage of crude hemolysin through ion-exchange columns that contained 6 M urea resulted in the appearance of only the cationic form. In a later publication, Wadstrom and Mollby (196) revised the pI to 9.4 ± 0.1. Table 9 summarizes information on the physical characteristics of beta hemolysin.

Mode of action. The mode of action of beta hemolysin has been discussed by Wiseman (211), Jeljaszewicz (97), and Bernheimer (8). Doery et al. (42, 43) first noted that beta hemolysin released acid-soluble phosphorus from

TABLE 7. *Purification of staphylococcal beta hemolysin*

Reference	Strain	Procedures[a]	Sp act (HU of protein or nitrogen/mg)
Robinson et al. (168)	L16	D, B, D, A	?
Jackson (92)	J32A	D, D, B	?
Chesbro et al. (27)	UNH-Donita	B	?
Wiseman (208)	R-1, 252F	B, D	10^6
Wiseman and Caird (213)	R-1, 252F	B, D, C	?
Maheswaran et al. (133)	J19	D, C, B, B	7×10^4
Gow and Robinson (75)	MB534	D, C, B, E	5×10^5
Haque and Baldwin (83)	681	D, B	10^7
Wadstrom and Mollby (196)	R-1	B, A[b], C	3×10^{11}
Bernheimer et al. (12)	G-128, R-1, 234	D, D, A[b]	2.6×10^5

[a] A, Electrophoresis (starch block); B, ion-exchange chromatography; C, gel filtration (molecular sieve); D, precipitation; E, electrophoresis (sucrose gradient).
[b] Electrofocusing (pH gradient).

erythrocytes, the source of which was sphingomyelin, a phospholipid widely distributed in mammalian cell membranes. This observation has since been confirmed by a number of investigators (63, 126, 129, 130, 196, 212, 213). Sphingomyelin is apparently not found in *S. aureus* (63) and beta hemolysin itself plays no demonstrable role in staphylococcal lipid metabolism. The degradation of sphingomyelin as originally suggested by Doery is thought to proceed as follows:

$$\text{Sphingomyelin} \xrightarrow[Mg^{2+}]{\text{beta hemolysin}} N\text{-acyl sphingosine} + \text{phosphorylcholine}$$

This observation is based on recovery of all phosphorus and half of the nitrogen in an aqueous extract of the reaction products and was confirmed by the identification of the end products by thin-layer chromatography.

Of several phospholipids tested, it was found that the rate of hydrolysis was most rapid with sphingomyelin (213). The beta hemolysin did not hydrolyze phosphatidylethanolamine, phosphatidylcholine, or phosphate bonds of ribonucleic acid, beta glycerophosphate and phenylphosphate. Doery et al. (43) observed that hemolysin also attacked lysophosphatidylcholine. It has been shown (213) that hemolytic sensitivity of erythrocytes to beta hemolysin is correlated with their sphingomyelin content.

Requirement of the beta hemolysin for Mg^{2+} ions has been documented by various workers (43, 75, 82, 130, 208, 213). Other metal cations such as Co^{2-} and Mn^{2+} also enhance hemolysis of erythrocytes in the presence of beta hemolysin (208). Maheswaran and Lindorfer (130) have investigated some aspects of the kinetics of hydrolysis of sphingomyelin by purified beta hemolysin. They found that release of acid-soluble phosphorus from erthrocyte ghosts by hemolysin is directly proportional to temperature between 37 and 45 C, although release was maximal at 41 C when purified sphingomyelin was the substrate. When hemolysin concentration was plotted against liberation of phosphorus, a straight line was obtained which indicated a first order reaction.

TABLE 8. *Amino acid analysis of staphylococcal beta hemolysin*

Amino acid	Residues	
	Bernheimer et al. (12)[a]	Fackrell (53)[b]
Aspartic acid	44	44
Threonine	14	17
Serine	23	33
Glutamic acid	25	38
Proline	10	0
Glycine	21	39
Alanine	12	25
Valine	12	18
Cystine/cysteine	0	4
Methionine	4	0
Isoleucine	9	17
Leucine	12	20
Tyrosine	14	1
Phenylalanine	8	13
Lysine	28	33
Histidine	8	8
Tryptophan	6	?
Arginine	6	13
Ammonia	?	143

[a] Methionine set at 4 in original data.
[b] Histidine set at 8.

The "hot-cold" reaction. Beta hemolysin, along with several similar agents (e.g., the alpha toxin of *Clostridium perfringens*) is a "hot-cold" hemolysin; that is, hemolysis is

TABLE 9. *Some characteristics of staphylococcal beta hemolysin*

Reference	Strain	$s_{20,w}$	Mol wt	pI	Enzyme activity
Gow and Robinson (75)	MB534	1.7	—[a]		?
Mollby and Wadstrom (145)	R-1	—	—	9.5 ± 0.1	?
Chesbro and Kucic (26)	UNH-15	—	1.55×10^4	—	?
Wadstrom and Mollby (196)	R-1	—	3.8×10^4	9.4 ± 0.1	Sphingomyelinase
Fackrell and Wiseman (56)	R-1	1.8	2.6×10^4[b] 1.61×10^4[c]	9.5	Sphingomyelinase
Bernheimer et al. (12)	G-128, R-1, 234	—	3×10^4	9.0 ± 0.1	Sphingomyelinase
Maheswaran and Lindorfer (132)		—		9.5	Sphingomyelinase
Chesbro et al. (27)	UNH-Donita	—	5.9×10^4	—	Carbohydrase (?)

[a] Not reported.
[b] Gel filtration.
[c] Amino acid analysis.

significantly increased if incubation at 37 C is followed by a period of holding at a lower temperature. Much has been written about this reaction, but it is probably true to say that it is still imperfectly understood. Wiseman (209, 211) discussed the phenomenon, finding that rapid alteration of pH or NaCl concentration in suspensions of erythrocytes treated with hemolysin caused intensified hemolysis at 37 C. He further suggested that a reduction in temperature may cause sudden contraction of the treated membrane which might break weak bonds and cause the structure to disintegrate.

Recently, Meduski and Hochstein (144) have advanced the hypothesis that the "hot-cold" phase results from changes in choline residues of membrane sphingomyelin. They found that the lytic effects of I_3^- ions on erythrocytes are similar to those observed in "hot-cold" lysis. The addition of dipalmitoyl lecithin to the system prevented I_3^- hemolysis of erythrocytes, provided it was added before initiation of the "hot" phase. It appeared that I_3^- bound to the lecithin as a stable complex, a triiodide, which was chromatographically identifiable. It would seem that I_3^- can also interact with $-N^+(CH_3)_3$ groups of other phospholipids at pH 7.0 which also leads to "hot-cold" lysis. The authors point out that the sphingomyelins may bind I_3^- most actively because of their physicochemical properties. Erythrocytes of sheep, man, and rats are, in that order, decreasingly sensitive to the action of beta hemolysin as they are to I_3^- hemolysin. Meduski and Hochstein thus argue that the fixed positive charge of phospholipids mediates leakage of hemoglobin through the membrane and that this leakage reaches "equilibrium" during the "hot" phase. A reduction in temperature would shift this equilibrium so that leakage is increased. Their view is that "hot-cold" hemolysis is a feature of the response of erythrocytes to any agent which affects $-N^-(CH_3)_3$ groups of membrane phospholipids. Bernheimer (8) has suggested that sphingomyelin, presumably located on or near the membrane surface, undergoes extensive hydrolysis in the presence of beta hemolysin to expose lipid monolayers. These monolayers withstand incubation at 37 C, but temperature reduction leads to thermodynamic instability with resulting collapse of multiple areas of the membrane and hemolysis.

Toxic properties of beta hemolysin. Most controversy centers on the toxicity of beta hemolysin. Observations have contradicted one another, although it is certainly true that some work has been done with impure preparations. A number of investigators have observed toxic effects on mammalian cell cultures and suspensions (27, 98, 116, 210). More recently, Wadstrom and Mollby (198) have reported that beta hemolysin is cytotoxic for HeLa cells, human fibroblasts, and human thrombocytes, although Thelestam et al. (189) did not confirm this finding for hemolysin acting on human fibroblasts. Contradictory results have been obtained by Gladstone and Yoshida (70), who found that crude hemolysin had no effect on a variety of cultured cells including HeLa, L, HL, FL, HEp, chick fibroblasts, and rat heart connective tissue cells. Hallander and Bengtsson (80) also did not observe toxicity when beta hemolysin was incubated with human, bovine, and monkey kidney tissue cells. Conflicting observations have been made of the effects of beta hemolysin injected into various animals. Heydrick and Chesbro (87) claimed that intraperitoneal injection of hemolysin into guinea pigs was lethal only if given with Mg^{2+} ions. Wiseman (208) found that partially purified preparations from two strains of *S. aureus* were not lethal for mice or rabbits whether or not Mg^{2+} ions were included in the

inoculum. Intradermal injections of the hemolysin into rabbits caused slight swelling with an erythematous flush, but no necrosis. Maheswaran et al. (133) could not demonstrate dermonecrosis in rabbits after intradermal injection of rabbits in the presence or absence of Mg^{2+}. Gow and Robinson (75) reported that the hemolysin killed rabbits when injected in doses of 40 to 60 μg. Wadstrom and Mollby (197, 198) observed that the LD_{50} dose was in the range 10 to 100 μg for mice, rabbits, and guinea pigs and 0.25 to 10 μg for chicken embryos. Their view is that the beta hemolysin on a weight basis is as toxic as alpha hemolysin. However, their results were not reported as micrograms of hemolysin per kilogram of body weight. By comparison, LD_{50} doses reported for alpha hemolysin are 0.68 (56) and 1 μg of protein (3), or in the range of 27 to 34 μg of protein/kg of mouse tissue. Thus for rabbits, mice, and guinea pigs, we must accept that beta hemolysin is 10 to 160 times less toxic than alpha hemolysin. Variations in the strain of mice employed, as well as differences in beta hemolysin preparations, could account for the observed discrepancies in findings. The basis for such conflicting reports continues to be elusive at the present time.

Delta Hemolysin

Production. Production of delta hemolysin on various media and the effect of CO_2 on titers have been reviewed by Wiseman (211) and Jeljaszewicz (97). Two methods have largely been favored: (i) cultivation on solid media overlain with cellophane and (ii) casein hydrolysate liquid media supplemented with yeast diffusate. The cellophane-on-agar technique (14) has been used with some variation in the composition of the medium by: Marks and Vaughan (139), Jackson and Little (95), Hoffman and Streitfeld (88), Murphy and Haque (152), and Wiseman and Caird (214). The semidefined medium of Bramann and Norlin (16) has been used with some variation by different investigators (86, 101, 108, 119, 219). Since Williams and Harper (207) observed that delta hemolysin was not produced in a liquid medium and that its production required no CO_2, more recent work has utilized aeration of liquid media with oxygen or air at a controlled rate in the presence or absence of CO_2.

Purification. A summary of the essential features of purification schemes is given in Table 10. It should be noted that the procedure of Yohida (219) yielded crystalline delta hemolysin contaminated with beta hemolysin and ribonuclease (70). It might also be pointed out that the one-step procedure of Kreger et al. (119) gives delta hemolysin contaminated with alpha and gamma hemolysins (53, 54).

Physicochemical characteristics. Delta hemolysin activity is not affected by ethylenediaminetetraacetic acid, citrate, or metal cations (95, 211). A property of the hemolysin which sets it apart from other hemolytic proteins of *S. aureus* is its inhibition by serum components (70, 95, 119, 135, 214). According to Kapral (105, 106), the phospholipid content of sera or crude protein fractions might account for the inhibitory activity. Maheswaran and Lindorfer (131) found that delta hemolysin could be fractionated into three components of pI 3.32 (delta I), 3.75 (delta II), and 8.45 (delta

TABLE 10. *Purification of staphylococcal delta hemolysin*

Reference	Strain	Procedures[a]	Sp act[b]
Jackson and Little (96)	1363, 2426, 2428, 2429	A, B, C,	3,200
Yoshida (219)	Foggie	(1) A, B, C, C, D, D	120
		(2) A, D, D	400
Caird and Wiseman (22)	E-delta	B, B, D, D	12,000
Kreger et al. (119)	Wood 46M[c]	D	400[d]
			200[e]
Heatley (86)	186×[f]	A, B, C, C, C, B	250–300
Kapral and Miller (108)	PG114	E, B	400[d]
			200[e]
Kantor et al. (101)	Wood 46M	D	75

[a] A, Heating; B, precipitation; C, extraction with organic solvent; D, ion-exchange chromatography; E, "Diaflo" ultrafiltration.

[b] Hemolytic units per milligram of protein or nitrogen.

[c] Derived from Wood 46 by ultraviolet irradiation.

[d] Insoluble delta hemolysin.

[e] Soluble delta hemolysin.

[f] Derived from Newman by subculture.

III) by electrofocusing. The results of Kreger et al. (119) differed from those of Maheswaran and Lindorfer in that two peaks of pI 9.5 ± 0.3 and 5.0 ± 0.2 were obtained. The 9.5 peak represented about 70% of the hemolytic activity, but in Maheswaran's preparation the 3.75 peak was the major portion. Kantor et al. (101) found one peak of pI 5.2 in the presence of 0.1% Tween 80. Hemolytic activity was equally spread between three peaks of pI 4.65, 6.7, and 9.0. The pI of the preparation of Mollby and Wadstrom (145) was 9.6 ± 0.2, identical with the value obtained by Fackrell and Wiseman (56). At any rate, the pI of a major proportion of delta hemolysin activity is in the range of 9 to 10. Various molecular weights have been reported (Table 11) ranging from 6.8×10^4 to greater than 2×10^5.

The structure of the delta hemolysin molecule has occupied the attention of Kantor et al. (101), who claimed that it consisted of five subunits weighing 21,000 daltons apiece as indicated by dissociation in 0.1% Tween 80. They stated that further treatment with detergent yielded smaller particles. It was thought that each fragment of 21,000 daltons represented a tetramer of identical polypeptide chains and that the so-called "native" form of the hemolysin was a structure which contained five of these tetramers oriented in a planar-radial configuration.

Hemolytic activity. Erythrocytes of various species are more uniformly sensitive to delta hemolysin than alpha, beta, or gamma hemolysins (97, 211).

Reaction kinetics. A linear relationship is observed between degree of hemolysis of human erythrocytes and delta hemolysin concentrations within roughly approximate limits of 15 to 75% lysis (86, 95, 219). Some studies have been made with phosphatidylinositol (211, 214). The reaction between delta hemolysin and the phospholipid was linear up to a hemolysin concentration of 500 hemolytic units/ml, the rate being directly proportional to temperature between 30 and 56 C. An activation energy of 18,750 cal (78,487.5 J) was obtained from an Arrhenius plot.

Mode of action. Several investigators have expressed the opinion that surface activity of the hemolysin accounts for lysis of sensitive erythrocytes (7, 8, 86, 118, 163). Bernheimer (8) has compared the activity of delta hemolysin to surfactin from *Bacillus subtilis*, a heptapeptide of which glutamic acid is the N terminus in amide linkage with 3-hydroxy-13-methyl-tetradecanoic acid. Although delta hemolysin and surfactin have some properties in common, others are dissimilar. Most striking of the differences are the discrepancies in molecular weight and in immunogenicity. Delta hemolysin has recently been shown to be immunogenic (54). Quantitative data on the surface activity of delta hemolysin are not available at present.

Wiseman and Caird (214) and Wiseman (211) have suggested that delta hemolysin acts enzymatically on erythrocytes. The main finding was that hemolysin from the Newman and E-delta strains of *S. aureus* liberated organic phosphorus from erythrocytes in direct proportion to their sensitivity. Degradation of a speci-

TABLE 11. *Some characteristics of staphylococcal delta hemolysin*

Reference	Delta hemolysin concn	pI	Mol wt	($s_{20,w}$)
Yoshida (219)	0.4%	—[a]	6.8×10^4	6.1
			7.4×10^8	
Hallander (78)	?		$>2 \times 10^5$	—
Mollby and Wadstrom (145)	?	9.6 ± 0.2	—	—
Maheswaran and Lindorfer (131)		(I) 3.32		
	?	(II) 3.75	—	—
		(III) 8.45		
Caird and Wiseman (22)	6.0 mg/ml	—	$>2 \times 10^5$	2.8
				9.8
Kreger et al. (119)	0.6%[b]	(I) 9.5 ± 0.3	—	1.9
		(II) 5.0 ± 0.2		4.9
				11.9
Kantor et al. (101)	?	(I) 4.65	1.03×10^5	6.04
		(II) 6.70	1.95×10^5	
		(III) 9.00		
Heatley (86)	6.0 mg/ml	—	—	4.9

[a] Not reported.

[b] Concentration applies to ultracentrifuge experiments; concentration in electrofocusing column was a total of 20 mg.

fic phospholipid in erythrocyte ghosts was not detected, although commercial preparations of phosphatidylinositol, phosphatidylserine, and phosphatidylcholine were preferentially hydrolyzed in that order. No action on sphingomyelin was observed. Thus, the source of organic phosphorus in the erythrocyte is in doubt, although the reaction kinetics are in keeping with an enzymatic degradation of phospholipid.

Several observers have noted that delta hemolysin clarified egg-yolk agar, much as the alpha toxin (lecithinase) activity of *Clostridium perfringens* does. This finding has yet to be explained, since many claim that delta hemolysin has no phospholipase activity (86, 106, 119, 163). Wiseman and Caird (214) stated that the hemolysin did not degrade phosphatidylcholine. Furthermore, various phospholipids, including phosphatidylcholine, inhibit its hemolytic activity.

At present, the mode of action of delta hemolysin is not entirely clear, and it would be helpful if a careful reinvestigation of purified preparations from a number of strains was made, preferably in the same laboratory. What is needed is a careful study of the surface activity of the hemolysin, its action on artificial lipid spherules, and its effect on the composition of erythrocyte ghosts.

Toxicity of delta hemolysin. Most agree that delta hemolysin has an injurious effect on a wide variety of cells in culture and on leukocytes (67, 68, 70, 93, 94). It is also said to disrupt bacterial protoplasts, spheroplasts, and lysosomes (118, 119). Wiseman (210) and Jeljascewicz (97) have reviewed the major findings.

Some variation has been noted in the effect of delta hemolysin on animals as indicated in Table 12. The values shown are difficult to compare since standard methods have not been applied in calculation of the end point. The LD_{50} dose of Kreger's delta hemolysin was calculated from this data to be of the order of 2 mg for mice or 7.17 mg for guinea pigs. Wadstrom and Mollby (197) reported the very large LD_{50} dose of 125 mg in mice. Lethal or dermonecrotic effects observed in such large doses as these are probably due to contamination with alpha hemolysin. At any rate, the lethal and dermonecrotic effects of delta hemolysin are considerably less than those of alpha hemolysin.

Immunogenicity of delta hemolysin. Several investigators have not been able to produce antibody against delta hemolysin (70, 79, 101). Gladstone and Yoshida (70) claimed that the hemolysin was neutralized by alpha and beta globulin fractions of serum but only to a limited extent by gamma globulin. Kapral (106) suggested that this neutralizing activity was due to the phospholipid content of the serum. Elek (50) and Kayser and Raynaud (110) were of the opinion that delta hemolysin is antigenic. Donahue (45) found that 105 human sera investigated contained equal amounts of delta hemolysin inhibitor. He suggested that serum lipoproteins, and not immunoglobulins, were responsible for the inhibitory activity. Inhibition by serum was also noted by Maniar et al. (135). More recently Fackrell and Wiseman (54) removed nonspecific inhibitors from serum by precipitation and ion-exchange methods. Antiserum to delta hemolysin, from which such

TABLE 12. *Effects of delta hemolysin in animals*

Reference	Animal	Dose (mg/kg)[a]	
		Lethal	Dermonecrotic[b]
Kreger et al. (119)	Mouse	110 (MLD)[c]	—[d]
	Rabbit	30 (MLD)	0.5–1.0
	Guinea pig	—	0.5–1.0
Kreger et al. (119)[e]	Rabbit	—	<1–1
	Guinea pig	—	<1–1
Gladstone (67)[f]	Mouse	>10 (LD_{50})	—
	Rabbit	—	0.005–0.5
Wadstrom and Mollby (198)	Rabbit	5000 (LD_{50})	—
Fackrell and Wiseman (56)	Mouse	4 (LD_{50})	0.1
	Guinea pig	4 (LD_{50})	0.1

[a] Calculated on the basis of average weights of 25 g for mice and 1 kg for rabbits.

[b] These doses cause dermonecrosis, but information regarding the minimum or 50% necrotic dose was not available.

[c] MLD, Minimum lethal dose.

[d] Not reported.

[e] Delta hemolysin supplied by Kapral (108).

[f] Supplied by Yoshida.

inhibitors were removed, neutralized hemolytic activity and precipitated it with the antigen.

Gamma Hemolysin

Although gamma hemolysin was decribed in 1938 by Smith and Price (178) and its existence confirmed by Marks (137), most investigators, until quite recently, accepted the view of Elek and Levy (51) and Elek (50) that the alpha-2, gamma, and delta hemolysins were identical. It now seems clear that Elek could not have detected gamma hemolysin on blood agar plates since its activity is apparently inhibited by agar. Reports by Jackson (90), Guyonnet et al. (77), Guyonnet and Plommet (76), Mollby and Wadstrom (146), Taylor and Bernheimer (186), and Fackrell and Wiseman (56) have demonstrated its existence as a hemolysin distinct from alpha, beta, and delta hemolysins.

Production. Gamma hemolysin has been produced in the laboratory by essentially the same techniques which have been applied to other staphylococcal hemolysins. The method of Birch-Hirschfeld (14), in which cells are grown on cellophane overlaying agar media, has been used by Jackson (90) and by Fackrell and Wiseman (55). Others have used the liquid CCY medium of Gladstone and Van Heyningen (68) with production of acceptable yields (76, 77, 146), or a CCY modification (186).

Optimal conditions of production have not yet been thoroughly investigated.

Aeration has been used in production by Guyonnet and Plommet (76) with a mixture of 25% CO_2 in oxygen, but Mollby and Wadstrom (146) found that active aeration lowered titers. Others (55) have found that highest titers were obtained when trays were incubated in a mixture of 10% CO_2 in air at pH 7.0 for 24 h at 37 C. Hemolysin production was minimal beyond the pH range of 6.0 to 8.0. They also found that it was produced late in logarithmic growth, and that comparatively small amounts were cell bound.

Purification. Methods are given in Table 13. Guyonnet et al. (77) and Guyonnet and Plommet (76) found that hemolytic activity was retained on a column of hydroxylapatite, but that a molar gradient of 0.1 to 0.6 M eluted two inactive peaks which, when combined, restored hemolytic activity. Taylor and Bernheimer (186) confirmed this finding, but neither Mollby and Wadstrom (146) nor Fackrell and Wiseman (55) were able to do so. The existence of two peaks of lytic activity may be related to the use of hydroxylapatite, since this material was not used by Mollby and Wadstrom or Fackrell and Wiseman.

Physicochemical characteristics. Jackson (90) inactivated gamma hemolysin by heating at 55 C for 10 min or with cysteine or ascorbic acid. He found that agar inhibited its activity. Guyonnet and Plommet (76) confirmed inhibition with agar but could not repeat Jackson's observations regarding ascorbic acid and cys-

TABLE 13. *Purification of staphylococcal gamma hemolysin*

Reference	Strain	Procedure[a]	Sp act[b]
Guyonnet et al. (77)	5R	A	1,000–2,500
Mollby and Wadstrom (146)	5R	A, B	?
Taylor and Bernheimer (186)	5R	C, A	4,000
Fackrell and Wiseman (55)	5R	C, D, E, F, E	10^5

[a] A, Ion-exchange chromatography; B, electrofocusing; C, ultrafiltration; D, gel filtration; E, precipitation; F, other (NaCl extraction).

[b] Hemolytic units per milligram of protein.

TABLE 14. *Some characteristics of staphylococcal gamma hemolysin*

Reference	Strain	$s_{20,w}$	pI	Mol wt	Inhibition by:	
					Agar	Phospholipids
Guyonnet and Plommet (76)	5R	—[a]	—	—	+	?
Mollby and Wadstrom (146)	5R	—	9.5	—	+	?
Taylor and Bernheimer (186)	5R	—	9.8[b]	2.6×10^4		
			9.9[c]	2.9×10^4	+	+
Fackrell and Wiseman (56)	5R	2.6	6.0	4.5×10^4	+	+

[a] Not reported.

[b] Component A.

[c] Component B.

teine. Some characteristics of the gamma hemolysin are given in Table 14. Noteworthy is its inhibition by agar and phospholipids. Isoelectric focusing of several preparations indicates a pI of 9.5 to 9.9, but the value obtained by Fackrell and Wiseman (56) was 6.0. Mollby and Wadstrom (146) found that diethylaminoethyl-Sephadex adsorbed gamma hemolysin in contrast to alpha, beta, and delta hemolysins, all of which are basic proteins, but this was not confirmed by Taylor and Bernheimer (186).

Fackrell and Wiseman (56) have obtained additional information on gamma hemolysin, as shown in Table 15, where it is compared to alpha, beta, and delta hemolysins.

Gamma hemolysin is antigenic (56, 76, 185).

Hemolytic activity. Gamma hemolysin acts on human, rabbit, and sheep but not horse erythrocytes (76). Mollby and Wadstrom (146) found that rabbit erythrocytes were most sensitive, whereas those of fowl were most resistant. Other species (sheep, goat, human, and dog) were of intermediate sensitivity, whereas horse cells were only slightly lysed. Fackrell and Wiseman (56) found that gamma hemolysin had the greatest affinity for rabbit and sheep cells and the least for chicken and pigeon cells. The hemolytic spectrum of gamma hemolysin is not particularly related to the spectra of the other staphylococcal hemolysins.

Mode of action. The mode of action of gamma hemolysin is unknown, but a few facts have recently become available. Hemolysin from the 5R strain is inhibited by ethylenediaminetetraacetic acid and citrate, and sodium ions appear to be required for lysis (56). There is no doubt that it is a protein (56, 186). Its reaction kinetics are compatible with those of an enzymatic reaction, evidence of a cation requirement supporting this contention. Other relevant findings are a pH optimum (with respect to hemolysis) at 7.0, temperature optimum between 37 and 45 C, and a reaction velocity directly proportional to hemolysin concentration over the range 0 to 10 hemolytic units/ml (56).

Phospholipids inhibit the hemolytic action of gamma hemolysin (186) and this has been supported by our own recent findings (56). The hemolysin released acid-soluble phosphorus and nitrogen from erythrocyte ghosts, but phospholipids derived from ghosts were unaffected. Phosphatidylserine, phosphatidylethanolamine, sphingomyelin, and phosphatidylinositol were not hydrolyzed by the hemolysin, nor did it attack tosyl arginine methylester or azocoll. However, membrane phospholipids competitively inhibited hemolysis in contrast to membrane proteins. These observations confirm the data of Taylor and Bernheimer (186), who also found that phospholipids of erythrocyte membranes were not attacked by gamma hemolysin.

Toxicity. Information concerning effects of gamma hemolysin in animals is scant. Smith and Price (178) originally reported that crude hemolysin killed rabbits but not mice and guinea pigs, although it was slightly dermonecrotic for the latter. Fackrell and Wiseman (56) have found that 100 μg of purified gamma hemolysin subcutaneously injected into guinea pigs and rabbits showed no effect. The same dose injected intraperitoneally into mice by the

TABLE 15. *Comparison of some properties of staphylococcal hemolysins*[a]

Property	Alpha	Beta	Gamma	Delta
Sedimentation constant ($s_{20,w}$)	1.4, 3.0	1.8	2.6	2.8, 9.8
Extinction coefficient (E_{280}^{1cm})	13.56	4.24	28.35	29.08
Molecular weight (gel filtration)	4.5×10^4	2.6×10^4	4.5×10^5	2×10^5
Isoelectric point (pI)	8.5	9.5	6.0	9.6
N-terminal amino acid	Histidine[b]	?	Methionine	Proline
Most sensitive erythrocytes	Rabbit	Sheep	Rabbit	Human
Cation requirement	—[c]	$Mg^{2+}/Co^{2+}/Mn^{2+}$	Na^+/K^+	—
Inhibition by ethylenediaminetetraacetate	—	+	+	—
Inhibition by phospholipids	+[d]	+	+	+[e]
Surface activity	+[f]	?	?	+[g]

[a] Data from Fackrell and Wiseman (56), Wiseman (208), and Wiseman and Caird (213–215).
[b] Inactive form.
[c] None.
[d] Freer et al. (61) and Arbuthnott et al. (5).
[e] Kapral (106).
[f] Freer et al. (61).
[g] Heatley (86) and Bernheimer (8).

intravenous route was also innocuous, but guinea pigs were killed in minutes if 50 μg was injected intracardially. Autopsy findings indicated massive hemorrhage of the kidney and serosal surfaces of the intestines accompanied by frank lysis of erythrocytes in major veins and arteries. Incubation of gamma hemolysin with cells of the C-6 (human lymphoblasts) line increased the rate at which the cells took up trypan blue. Optical densities of suspensions of rabbit and human platelets and human leukocytes were decreased in the presence of gamma hemolysin.

Epsilon Hemolysin

Elek and Levy (51) claimed that 95% of 77 coagulase-negative skin strains of staphylococci produced what Elek (50) called a "wide-zone" hemolysin, which they designated "epsilon." Epsilon hemolysin produced a wide zone of lysis on rabbit and sheep blood agar plates and was not neutralized by staphylococcal antitoxin, which presumably would contain antibody to alpha, beta, gamma, and delta hemolysins. Marks (138) also studied a number of coagulase-negative strains, finding no evidence that they were hemolytic. Several investigators suggested that coagulase-negative strains may produce delta hemolysin (68, 138, 141), and Kleck and Donahue (114) claim to have demonstrated that epsilon and delta hemolysins are identical. Ali and Haque (2) and Cabrera and Haque (21) applied their electrophoresis localization technique to the detection of hemolysins produced by *S. epidermidis*. Several hemolysins were identified (epsilon, theta, and kappa hemolysins), but their significance is not understood at present. Wadstrom et al. (199) have studied 20 or more strains of *S. epidermidis* which commonly produced alpha and delta hemolysins, but detected no other hemolysins.

Role of the Hemolysins in Pathogenicity and Virulence of Staphylococci

While most investigators would agree that coagulase production by staphylococci is closely associated with disease-causing potential, some have argued that the presence of alpha hemolysin is a more accurate indication of pathogenicity (28, 138, 171). Lack and Wailling (122) suggested that pathogenicity of *S. aureus* is more likely to be correlated with a broad spectrum of toxins than with a single substance after finding that only 82% of 435 strains produced alpha hemolysin. Elek and Levy (51), in their classical study of 200 coagulase-positive *Staphylococcus* strains from human sources, found that 96% produced alpha hemolysin, 11% produced beta hemolysin, and 97% produced delta hemolysin. None failed to produce hemolysin on blood agar plates, although gamma hemolysin was unlikely to have been detected. The alpha-delta hemolysin combination was the most common in human strains and alpha-beta-delta hemolysin production was the favored combination in strains from animal sources.

Burns and Holtman (20), in their investigation of about 700 strains of coagulase-positive staphylococci in two studies, found that no single factor could be identified specifically with virulent strains. However, they did observe that coagulase and deoxyribonuclease production was more characteristic of strains from suppurative lesions than other strains. In 221 isolates, Noble (154) found that production of 10 extracellular substances apparently had no correlation with virulence, but there was evidence that beta hemolysin and staphylokinase were implicated in the initiation of lesions. These is nevertheless some indication that extracellular enzyme formation may be greater in strains from lesions than in those from carriers. Bhaskaran and Jayakar (13) examined 186 coagulase-positive organisms isolated from infections and 100 from healthy carriers. Hemolysin production (alpha, beta, and delta), and that of several other "aggressins," was elevated in the isolates from lesions.

Several investigators have been concerned with in vivo production of the hemolysins. Gladstone and Glencross (69) observed that high yields of alpha hemolysin were produced by coagulase-positive strains from human sources grown for 24 h in cellophane sacs in rabbits. These in vivo hemolysin-producing strains were inactive when tested on the appropriate blood agar. Coagulase-negative strains were uniformly nonhemolytic. They pointed out that in vitro hemolysin testing may not always be valid and that the imperfect correlation of in vitro hemolysin production with virulence might be misleading. Hauser and Berry (85), Kapral et al. (109), and Cybulska and Jeljaszewicz (34) made similar observations. Thus, evidence is conflicting with regard to the role alpha hemolysin plays in staphylococcal disease. A number of investigators have argued for the importance of alpha hemolysin as a virulence factor (59, 60, 104, 179, 184) and others have refuted it (6, 58).

Kapral (107) has reviewed his work in which mice and rabbits were used in the study of experimental infections. He found that intraperitoneal injection of not less than 2×10^8 non-

encapsulated staphylococci which contained bound coagulase caused formation of clumps of cocci and leukocytes after several hours. Clumped inocula, which produced a lethal dose of alpha hemolysin (15 to 20 HD_{50}) or delta hemolysin (200 HD_{50}) within 2 h of injection before they were surrounded by leukocytes, caused death in 6 to 8 h. Kapral considered alpha hemolysin to be of major importance in this experimental model. Delta hemolysin became a key factor only if alpha antitoxin was present or if the organisms did not produce sufficient quantities of alpha hemolysin. He also felt that leukocyte infiltration of the clumps of cocci reduced absorption of alpha hemolysin, since some strains of mice that were unable to mount a sufficient leukocyte response were more quickly killed by smaller numbers of organisms. A further observation was that abscesses excised from the mouse peritoneal cavity contained alpha hemolysin concentrations beyond those required to kill the animal; yet it survived for days provided that the numbers of cocci did not increase.

In human infections, the picture is less clear and it is easy to suggest that the necrotic manifestations of disease in man are due to the hemolysins, some of which are known to cause such reactions in the skin of laboratory animals. In fact, there is scant evidence that this is so. Elek (49) and Elek and Conen (52) have performed direct virulence tests of staphylococci in human volunteers. They showed that actual multiplication of cocci is required after intradermal injection if pus is to be formed and that injection of killed organisms at the same dosage produced no pus. The minimum pus-forming dose was the same whether or not the organisms were injected as a washed suspension, which suggested that the presence of preformed hemolysin had little effect on development of the lesion. Smaller numbers of organisms inoculated together with larger amounts of hemolysin did not produce pus where less than the minimum pus-forming dose was administered. Injection of the larger amount of hemolysin alone, however, caused painful swelling and some systemic disturbance. Elek and his co-workers found that when a number of staphylococcal strains of presumed differences in virulence were tested in this way the pus-forming dose was always about 1 to 5 million cocci. These same strains showed varying virulence for rabbits. The doses required were uniformly higher in rabbits and these animals are extraordinarily sensitive to alpha hemolysin.

Elek's results, considered together with those of Kapral, point to the inherent difficulties in this kind of investigation and certainly suggest that the most cautious interpretation is required of animal experiments meant to serve as a model for human infection. Another major problem in these studies is that it is difficult, if not impossible, to exclude the influence of staphylococcal products other than the one under investigation. Rather than inject crude culture filtrates or live cocci, some individuals have favored the use of purified preparations. Although this approach may at first glance seem the most plausible, its detractors contend that it is an artificial one and does not resemble the natural infection in which many factors presumably interact. While each approach has its advocates, we are in fact trapped in a circular argument from which at present there is no escape.

Some indication of the importance of the hemolysins in pathogenesis of staphylococcal disease might be gained from an examination of antitoxin therapy in man. Although it is not within the scope of this review to discuss in detail this question, a few points are relevant. Antitoxin therapy for staphylococcal disease was fashionable for some years, until the advent of penicillin. Even so, evidence of its efficacy was conflicting (50). It is probably true to say that about 75% of adults have antibody to alpha hemolysin, but titers vary considerably. The diagnostic value of such antibodies in sera of patients is questionable, although it has been said that they are of some significance in bone and joint infections. Lack and Towers (123), who measured four antibodies produced during the course of these infections, found that Panton-Valentine (PV) antileucocidin titers arose more constantly than alpha antihemolysin. In this connection, Taylor and Plommet (184) have shown that gamma hemolysin is antigenic in man; that gamma titers are rather higher than those of alpha antihemolysin; and that they are more consistently observed in patients with osteomyelitis. They suggest that determination of gamma antihemolysin titers is useful in diagnosis of the infection and in following the effects of treatment. Mudd (150) and Mudd et al. (151) have advocated the use of alpha hemolysin toxoid and Panton-Valentine leucocidin toxoid on the basis of studies in chronically ill and other patients. This view has met with some resistance by other investigators (169).

Beta antihemolysin has been demonstrated in a number of human patients (45, 46, 166, 192). Uyeda et al. (191) found antibody to beta hemolysin in all 165 persons tested. The relationship of beta and gamma hemolysins to disease in man and other animals is less clear

than the question of alpha hemolysin, although antibodies to beta hemolysin are of widespread occurrence. It is tempting to speculate that an enzyme like beta hemolysin would offer an advantage to the *Staphylococcus* in permitting it to gain a foothold in a wide variety of tissues in which sphingomyelin is a common membrane constituent. Disruption of the membranes would release metabolites required by the bacteria. Slanetz and Bartley (177) have observed that beta hemolysin production is particularly characteristic of strains of staphylococci associated with bovine mastitis and one could ask whether the skin and tissues of the teat might be richer in sphingomyelin than those of other body areas.

The Use of Staphylococcal Hemolysins in Membrane Structural Studies

The usefulness of bacterial toxins as tools of membrane research was largely unappreciated until quite recently when investigations into the effect of such purified agents on membrane structure and function were more widely publicized. It is not the purpose of this review to offer detailed analysis of this field, since the question has been dealt with by others (8), but rather to give some indication of the value of staphylococcal hemolysins in membrane research and to provide some guidance to the pertinent literature.

Alpha and delta hemolysins. As stated elsewhere in this review, alpha hemolysin causes the formation of rectangular arrays of ringed structures on the surfaces of sensitive erythrocyte membranes. These arrays were abolished if the membranes were pretreated with *C. perfringens* phospholipase C (165). This was interpreted to indicate that membrane phospholipid exists as micelles arranged in a rectilinear lattice.

Bernheimer et al. (11), however, found that the rectangular ordering frequently seen in hemolysin-treated erythrocyte membranes by themselves and others (61, 165) may result from the interaction of membranes and alpha hemolysin contaminated with delta hemolysin, the staining technique itself, or both (Fig. 1). Bernheimer et al. showed that the use of ammonium molybdate rather than neutral sodium phosphotungstate as a negative stain yielded a random distribution of the rings in the presence of alpha hemolysin, but it is important to note that addition of delta hemolysin promoted reordering of the rectangular array of rings. Thus the findings of Remsen et al. (165) may require re-interpretation.

There is some evidence that these rings are 12*S* alpha hemolysin, because treatment of ring-structured membranes with urea liberates hemolytic activity (8). It is noteworthy that the rings seen with so many species of mammalian cells are not observed on bacterial membranes. This must reflect some basic difference in membrane structure or organization between the two kinds of cells.

Beta hemolysin. This hemolysin might be regarded as the most useful of the staphylococcal hemolysins in membrane research because it is an enzyme with a known mode of action. Beta hemolysin, as we have noted, principally hydrolyzes sphingomyelin, a phospholipid widely distributed in mammalian cell membranes, with the liberation of *N*-acyl sphingosine (ceramide) and phosphorylcholine. Colley et al. (30) have investigated the interaction of beta hemolysin with human and porcine erythrocytes. They observed that initially degradation of 75 to 80% of the substrate occurred in the absence of hemolysis, but repetition of the experiment in the presence of nonhemolytic *B. cereus* phospholipase C caused complete hemolysis of the erythrocytes. It seemed that beta hemolysin must first hydrolyze sphingomyelin and that removal of phosphorylcholine was a precondition of the interaction of *B. cereus* enzyme with its phosphoglyceride substrate, leading to lysis. One can thus speculate on the arrangement and distribution of these phospholipids in such membranes. In contrast, Součková and Soucék (181) found that beta hemolysin could not lyse sheep erythrocytes if they had first been treated with *Corynebacterium* phospholipase D. Their interpretation was that removal of choline from sphingomyelin by phospholipase D made the membranes resistant to beta hemolysin. Alternatively, phospholipase D may interfere with access of beta hemolysin to its substrate.

Bernheimer et al. (12) have studied the effect of beta hemolysin on erythrocytes by phase-contrast and electron microscopy. Sheep erythrocytes treated with beta hemolysin and examined by phase contrast were reduced in size and exhibited numerous phase-dense particles which varied in size. Further examination of this phenomenon by electron microscopy suggested that the particles were in fact membranous vesicles that contained amorphous material. Bernheimer was uncertain whether this material might be ceramide. Low et al. (126) have independently studied the action of beta hemolysin on human and bovine erythrocyte ghosts. Ultrastructural changes in these membranes, as observed by freeze etching after treatment with hemolysin, showed that amor-

phous material was deposited in the hydrophobic (inner) area of the bilayers. Low et al. took the view that this material might be insoluble ceramide and that the sphingomyelin substrate was located in the outer half of the bilayer.

These observations again raise the question of "hot-cold" hemolysis so characteristic of this agent. Bernheimer (8) has suggested that hydrolysis of sphingomyelin leaves areas of lipid deployed as a monolayer stable at 37 C. A reduction in temperature would lead to "thermodynamic instability" of this layer, resulting in membrane collapse and hemolysis. He felt that this explanation accounts for "hot-cold" lysis and the appearance of vesicles on the inside of sensitive erythrocyte membranes. It will be necessary to integrate this view with the explanation of Meduski and Hochstein (144) that the "hot-cold" phase results from changes in choline residues of sphingomyelin.

CONCLUSIONS

The hemolysins of *S. aureus* have been considered in terms of their physicochemical characterization and mode of action. There has been much progress, but major questions remain. With regard to chemical and biological characterization, earlier work with crude or partially purified preparations must be carefully reviewed. However, more recent studies still indicate disconcerting variation in properties which is in all likelihood the result of strain differences. Even though Wood 46 alpha hemolysin has been widely examined, published data are not uniform. Comparative studies of hemolysin produced by different strains are required (see Goode and Baldwin, 73). Other sources of variation are the way in which the hemolysin is handled as it is purified and how it is produced. It is certainly true that reported differences in properties can be due to artifacts created by the purification process. A uniform approach to these problems is needed, particularly in an organism as notoriously variable as the *Staphylococcus*.

Specific questions arise in consideration of the mechanism of action of alpha, gamma, and delta hemolysins. A popular view has been that surface activity of alpha and delta hemolysins accounts for their hemolytic activity, which is the result of interactions between hemolysin and phospholipids in the membrane. An alternative view attributes activity of these hemolysins to their enzymatic properties. At the present time it is not clear which view is correct, but there need not necessarily be conflict between these hypotheses. It is quite possible that a proteinase or phospholipase can be surface active or that interaction with phospholipids is the first step in a quite different sequence of reactions. It is well known that proteins and lipids display some degree of association when brought together (23). At any rate, few attempts have been made to correlate the erythrocyte hemolytic spectrum with a particular activity or characteristic of the hemolysins.

Gamma hemolysin is now well established as a fourth hemolysin, but its mode of action is unclear. There has been a heavy reliance on the 5R strain, and gamma hemolysin isolated from other strains of *S. aureus* must be investigated before firm conclusions are drawn.

A long list of toxic effects and metabolic interference has been compiled for the staphylococcal hemolysins in relation to their effects on animals and cultured mammalian cells. Such studies do not get at the basic problem of how the hemolysins interact with their substrate in sensitive cells, nor do they necessarily contribute to the question of pathogenicity.

The role of the hemolysins in the pathogenesis of staphylococcal disease remains imperfectly understood. It is nevertheless true that the hemolysins cannot be identified with the elusive "virulence factor" discussed by Abramson (1). Perhaps it is best to take the view of Lack and Wailling (122) that virulence in staphylococci is associated not with a single factor but with a broad spectrum of aggressins.

LITERATURE CITED

1. Abramson, C. 1972. *In* J. O. Cohen (ed.), The staphylococci, chapt. 9. Wiley-Interscience, New York.
2. Ali, M. I., and R. Haque. 1972. Identification of staphylococcal epsilon hemolysin. Can. J. Microbiol. 18:535–536.
3. Arbuthnott, J. P. 1970. Microbial toxins, vol. 3, chapt. 5. Academic Press Inc., New York.
4. Arbuthnott, J. P., J. H. Freer, and A. W. Bernheimer. 1967. Physical states of staphylococcal alpha toxin. J. Bacteriol. 94:1170–1177.
5. Arbuthnott, J. P., J. H. Freer, and B. Billcliffe. 1973. Lipid-induced polymerization of staphylococcal alpha toxin. J. Gen. Microbiol. 75:309–319.
6. Bartell, P. F., T. E. Orr, A. Geffen, and P. Iorio. 1968. Experimental infection of mice with *Staphylococcus aureus*: evidence against alpha-toxin and the terminal size of the bacterial population as determinants of lethality. J. Infect. Dis. 118:481–490.

7. Bernheimer, A. W. 1970. Microbial toxins, vol. 1, chapt. 5. Academic Press Inc., New York.
8. Bernheimer, A. W. 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta 344:27-50.
9. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. 30:455-468.
10. Bernheimer, A. W., and L. L. Schwartz. 1965. Effects of staphylococcal and other bacterial toxins on platelets *in vitro*. J. Pathol. Bacteriol. 89:209-223.
11. Bernheimer, A. W., K. S. Kim, C. C. Remsen, J. Antanavage, and S. W. Watson. 1972. Factors affecting interaction of staphylococcal alpha toxin with membranes. Infect. Immun. 6:636-642.
12. Bernheimer, A. W., L. S. Avigad, and K. S. Kim. 1974. Staphylococcal sphingomyelinase (beta hemolysin). Ann. N.Y. Acad. Sci. 236:292-306.
13. Bhaskaran, C. S., and P. A. Jayakar. 1969. Biochemical activity of coagulase positive staphylococci isolated from cases and carriers. Pathol. Microbiol. 33:39-43.
14. Birch-Hirschfeld, L. 1934. Ueber die Wirksamkeit der Extracts von auf zellophanagar gezuchteten Staphylokokken. Z. Immunitaetsforsch. Allerg. Klin. Immunol. 81:260-285.
15. Bonventre, P. F. 1970. Microbial toxins, vol 1, chapt. 2. Academic Press Inc., New York.
16. Bramann, J., and G. Norlin. 1951. A factor in yeast dialysate of significance in the formation of *Staphylococcus* toxin. Acta Pathol. Microbiol. Scand. 29:127-131.
17. Buckelew, A. R., and G. Colacicco. 1971. Lipid monolayers. Interactions with staphylococcal alpha-toxin. Biochim. Biophys. Acta 233:7-16.
18. Burnet, F. M. 1929. The exotoxins of *Staphylococcus pyogenes aureus*. J. Pathol. Bacteriol. 32:717-734.
19. Burnet, F. M. 1930. The production of staphylococcal toxin. J. Pathol. Bacteriol. 33: 1-16.
20. Burns, J., and D. F. Holtman. 1960. Biochemical properties of virulent and avirulent staphylococci. Ann. N. Y. Acad. Sci. 88: 1115-1124.
21. Cabrera, G., and R. Haque. 1973. Types of hemolysins produced by *Staphylococcus epidermidis*. Bacteriol. Proc., p. 118.
22. Caird, J. D., and G. M. Wiseman. 1970. Purification of the delta toxin of *Staphylococcus aureus*. Can. J. Microbiol. 16:703-708.
23. Camejo, G., G. Colacicco, and W. M. Rapport. 1968. Lipid monolayers: interactions with the apoprotein of high density plasma lipoprotein. J. Lipid Res. 9:562-569.
24. Cassidy, P. S., and S. Harshman. 1973. The binding of staphylococcal 125 I-alpha-toxin (B) to erythrocytes. J. Biol. Chem. 248: 5545-5546.
25. Cassidy, P. S., H. R. Six, and S. Harshman. 1974. Biological properties of staphylococcal alpha-toxin. Biochim. Biophys. Acta 332: 413-423.
26. Chesbro, W. R., and V. Kucic. 1971. Beta hemolysin: a persistent impurity in preparations of staphylococcal nuclease and enterotoxin. Appl. Microbiol. 22:233-241.
27. Chesbro, W. R., F. P. Heydrick, R. Martineau, and G. N. Perkins. 1965. Purification of staphylococcal beta hemolysin and its action on staphylococcal and streptococcal cell walls. J. Bacteriol. 89:378-389.
28. Christie, R., E. A. North, and B. J. Parkin. 1946. Criteria of pathogenicity in staphylococci. Aust. J. Exp. Biol. Med. Sci. 24: 73-78.
29. Colacicco, G., and A. R. Buckelew. 1971. Lipid monolayers: influence of lipid film and urea on the surface activity of staphylococcal alpha-toxin. Lipids 6:546-553.
30. Colley, C. M., R. F. A. Zwall, B. Roelofsen, and L. L. M. Van Deenen. 1973. Lytic and non-lytic degradation of phospholipids in mammalian erythrocytes by pure phospholipases. Biochim. Biophys. Acta 307: 74-82.
31. Cooper, L. Z., M. A. Madoff, and L. Weinstein. 1966. Heat stability and species range of purified staphylococcal alpha toxin. J. Bacteriol. 91:1686-1692.
32. Coulter, J. R. 1966. Production, purification, and composition of staphylococcal alpha toxin. J. Bacteriol. 92:1655-1662.
33. Coulter, J. R., and T. M. Mukherjee. 1971. Electron microscopic localization of alpha toxin within the staphylococcal cells by ferritin-labeled antibody. Infect. Immun. 4: 650-655.
34. Cybulska, J., and J. Jeljaszewicz. 1969. Staphylococcal interference. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B 211:186-197.
35. Czerski, P., J. Jeljaszewicz, S. Szmigielski, and C. Zak. 1968. Blastoid transformation of rabbit lymphocytes by staphylococcal alpha-hemolysin and leukocidin. Folia Haematol. (Frankfurt am Main) 90:328-335.
36. Dalen, A. B. 1973. Production of staphylococcal alpha toxin in a defined medium and identification of a stimulating factor from yeast extract. J. Gen. Microbiol. 74:53-60.
37. Dalen, A. B. 1973. The induction of staphylococcal alpha toxin by histidine. J. Gen. Microbiol. 74:61-69.
38. Dalen, A. B. 1973. The influence of pH and histidine dipeptides on the production of staphylococcal alpha toxin. J. Gen. Microbiol. 79:265-274.
39. de Christmas, M. J. 1888. Recherches experimentales sur la suppuration. Ann. Inst. Pasteur Paris 2:469-478.
40. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics, of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem.

Biophys. 100:19–130.
41. Doery, H. M., and E. A. North. 1961. The interaction of staphylococcal toxin and ganglioside. I. Inactivation of the lethal effect of staphylococcal toxin in mice. Aust. J. Exp. Biol. Med. Sci. 39:333–344.
42. Doery, H. M., B. J. Magnusson, I. M. Cheyne, and J. Gulasekharam. 1963. A phospholipase in staphylococcal toxin which hydrolyzes sphingomyelin. Nature (London) 198:1091–1092.
43. Doery, H. M., B. J. Magnusson, J. Gulasekharam, and J. E. Pearson. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40:283–296.
44. Dolman, C. E., and R. J. Wilson. 1940. The kitten test for *Staphylococcus* enterotoxin. Can. J. Public Health 31:68–71.
45. Donahue, J. A. 1969. Antistaphylococcal hemolysins and delta hemolysin inhibitor in adult human serum. Can. J. Microbiol. 15:957–959.
46. Donahue, J. A. 1969. Biological characteristrics of staphylococcal heat stable hemolysin. Bacteriol. Proc., p. 93.
47. Duncan, J. L., and G. J. Cho. 1971. Production of staphylococcal alpha toxin. I. Relationship between cell growth and toxin formation. Infect. Immun. 6:456–461.
48. Duncan, J. L., and G. J. Cho. 1972. Production of staphylococcal alpha toxin. II. Glucose repression of toxin formation. Infect. Immun. 6:689–694.
49. Elek, S. D. 1956. Experimental staphylococcal infections in the skin of man. Ann. N. Y. Acad. Sci. 65:85–89.
50. Elek, S. D. 1959. *Staphylococcus pyogenes* and its relation to disease. Churchill Livingstone, Edinburgh.
51. Elek, S. D., and E. Levy. 1950. Distribution of hemolysis in pathogenic and non-pathogenic staphylococci. J. Pathol. Bacteriol. 62:541–554.
52. Elek, S. D., and P. E. Conen. 1957. The virulence of *Staphylococcus pyogenes* for man. A study of the problems of wound infection. Br. J. Exp. Pathol. 38:573–586.
53. Fackrell, H. B. 1973. Studies with gamma hemolysin of *Staphylococcus aureus*. Ph.D. thesis, Univ. of Manitoba, Winnipeg, Canada.
54. Fackrell, H. B., and G. M. Wiseman. 1974. Immunogenicity of the delta hemolysin of *Staphylococcus aureus*. J. Med. Microbiol. 7:411–414.
55. Fackrell, H. B., and G. M. Wiseman. 1975. Production and purification of the gamma hemolysin of *Staphylococcus aureus* "Smith 5R." J. Gen. Microbiol., in press.
56. Fackrell, H. B., and G. M. Wiseman. 1975. Properties of the gamma hemolysin of *Staphylococcus aureus* "Smith 5R." J. Gen. Microbiol., in press.
57. Forlani, L., A. W. Bernheimer, and E. Chiancone. 1971. Ultracentrifugal analysis of staphylococcal alpha toxin. J. Bacteriol. 106:138–142.
58. Foster, E. A. 1963. The role of alpha-hemolysin in the pathogenicity of *Staphylococcus aureus*. J. Pathol. Bacteriol. 86:535–540.
59. Foster, E. A. 1965. Hemolysin production in the development of staphylococcal lesions. Science 149:1395–1396.
60. Foster, E. A. 1967. Tissue injury by toxins in experimental staphylococcal infections. Effect of polyvalent antitoxin on developing renal infections. Am. J. Pathol. 51:913–928.
61. Freer, J. H., J. P. Arbuthnott, and A. W. Bernheimer. 1968. Interaction of staphylococcal alpha toxin with artificial and natural membranes. J. Bacteriol. 95:1153–168.
62. Freer, J. H., J. P. Arbuthnott, and B. Billcliffe. 1973. Effects of staphylococcal alpha toxin on the structure of erythrocyte membranes: a biochemical and freeze-etching study. J. Gen. Microbiol. 75:321–332.
63. Fritsche, D. 1970. Untersuchungen zur Funktion des beta-Hämolysins von *Staphylococcus aureus*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1: Orig. Reihe B 213:348–355.
64. Galanti, B., F. Paradisi, A. Mancini, and G. Guisti. 1968. An early effect of *Staphylococcus* alpha-toxin on cells growing in culture: ATP levels in normal and intoxicated cells. Pathol. Microbiol. 32:277–284.
65. Ganczarski, A. 1962. Lodz. Tow. Nauk. Pr. Wydz. 4. 44:1–84.
66. Gladstone, G. P. 1938. The production of staphylococcal alpha haemolysin in a chemically-defined medium. Br. J. Exp. Pathol. 19:208–226.
67. Gladstone, G. P. 1966. Staphylococcal haemolysins. Postepy Mikrobiol. 5:145–160.
68. Gladstone, G. P., and W. E. Van Heyningen. 1957. Staphylococcal leucocidins. Br. J. Exp. Pathol. 38:123–137.
69. Gladstone, G. P., and E. J. Glencross. 1960. Growth and toxin production of staphylococci in cellophane sacs *in vitro*. Br. J. Exp. Pathol. 41:313–333.
70. Gladstone, G. P., and A. Yoshida. 1967. The cytopathic action of purified staphylococcal delta hemolysin. Br. J. Exp. Pathol. 48: 11–19.
71. Glenny, A. T., and N. F. Stevens. 1935. *Staphylococcus* toxins and antitoxins. J. Pathol. Bacteriol. 40:201–210.
72. Goode, R. L. and J. N. Baldwin. 1973. Purification of staphylococcal alpha toxin by electrofocusing. Prep. Biochem. 3:349–361.
73. Goode, R. L., and J. N. Baldwin. 1974. Comparison of purified alpha-toxins from various strains of *Staphylococcus aureus*. Appl. Microbiol. 28:86–90.
74. Goshi, K., L. E. Cluff, and P. S. Norman. 1963. Studies on the pathogenesis of staphylococcal infection. V. Purification and characterization of staphylococcal alpha hemolysin. Bull. Johns Hopkins Hosp. 112:15–30.

75. Gow, J. A., and J. Robinson. 1969. Properties of purified staphylococcal beta hemolysin. J. Bacteriol. 97:1026–1032.
76. Guyonnet, F., and M. Plommet. 1970. Hemolysine gamma de *Staphylococcus aureus*: purification et proprietes. Ann. Inst. Pasteur Paris 118:19–33.
77. Guyonnet, F., M. Plommet, and C. Bouillanne. 1968. Purification de l'hemolysine gamma de *Staphylococcus aureus*. C. R. Acad. Sci. 267:1180–1182.
78. Hallander, H. O. 1963. Fractionation of staphylococcal toxins by gel-filtration. Acta Pathol. Microbiol. Scand. 59:543–552.
79. Hallander, H. O. 1968. Characterization and partial purification of staphylococcal delta-lysin. Acta Pathol. Microbiol. Scand. 72: 586–600.
80. Hallander, H. O., and S. Bengtsson. 1967. Studies on the cell toxicity and species specificity of purified staphylococcal toxins. Acta Pathol. Microbiol. Scand. 70:107–119.
81. Haque, R. 1963. Purification and properties of staphylococcal beta hemolysin. Ph.D. thesis, Ohio State Univ., Columbus.
82. Haque, R., and J. N. Baldwin. 1964. Purification and properties of staphylococcal beta-hemolysin. I. Production of beta-hemolysin. J. Bacteriol. 88:1304–1309.
83. Haque, R., and J. N. Baldwin. 1969. Purification and properties of staphylococcal beta-hemolysin. II. Purification of beta-hemolysin. J. Bacteriol. 100:751–759.
84. Harvie, N. R. 1974. De-esterification of cholesteryl esters in human plasma alpha-lipoprotein (HDL) by preparations of staphylococcal alpha toxin. Biochem. Biophys. Res. Commun. 61:1283–1288.
85. Hauser, E. D., and L. J. Berry. 1961. The pathogenesis of *Staphylococcus* infection. I. The use of diffusion chambers in establishing the role of *Staphylococcus* toxins. J. Infect. Dis. 109:24–30.
86. Heatley, N. G. 1971. A new method for the preparation and some properties of staphylococcal delta hemolysin. J. Gen. Microbiol. 69:269–278.
87. Heydric, F. P., and W. R. Chesbro. 1962. Purification and properties of staphylococcal beta toxin. Bacteriol. Proc., p. 63.
88. Hoffman, E. M., and M. M. Streitfeld. 1965. The antibiotic activity associated with preparations of delta hemolysin of *Staphylococcus aureus*. Can. J. Microbiol. 11:203–211.
89. Holme, T., and T. Wadstrom. 1971. A continuous process for the production of extracellular proteins by *S. aureus*. Acta Pathol. Microbiol. Scand. 79:414–420.
90. Jackson, A. W. 1963. Staphylococcal gamma lysin and its differentiation from delta lysin. *In* N. E. Gibbons (ed.), Recent progress in microbiology: symposia. University of Toronto Press, Toronto.
91. Jackson, A. W. 1963. Purification of staphylococcal alpha-lysin. Can. J. Biochem. Physiol. 41:219–229.
92. Jackson, A. W. 1963. Purification of staphylococcal beta-lysin. Can. J. Biochem. Physiol. 41:755–761.
93. Jackson, A. W., and R. M. Little. 1956. The delta-toxin of *Micrococcus pyogenes* var. *aureus*. Bacteriol. Proc., p. 88.
94. Jackson, A. W., and R. M. Little. 1957. Leucocidal effect of staphylococcal delta lysin. Can. J. Microbiol. 3:101–102.
95. Jackson, A. W., and R. M. Little. 1958. Staphylococcal toxins. II. Factors affecting hemolysis by delta lysin. Can. J. Microbiol. 4:435–444.
96. Jackson, A. W., and R. M. Little. 1958. Staphylococcal toxins. III. Partial purification and some properties of delta lysin. Can. J. Microbiol. 4:453–461.
97. Jeljaszewicz, J. 1972. *In* J. O. Cohen (ed.), The staphylococci, chapt. 10. Wiley-Interscience, New York.
98. Jeljaszewicz, J., S. Szmigielski, M. Korbecki, and C. Zak. 1965. Histochemical demonstration of changes in enzymatic activity of KB cells produced by staphylococcal alpha and beta hemolysins. J. Infect. Dis. 115:421–428.
99. Jeljaszewicz, J., S. Szmigielski, and C. Zak. 1969. Distribution of ^{131}I-labelled staphylococcal alpha-hemolysin in the rabbit. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1: Orig. Reihe B 209:310–314.
100. Kadlec, O., and I. Seferna. 1972. Analysis of staphylococcal alpha toxin action in the *taenia coli* of the guinea pig. Toxicon 10: 465–472.
101. Kantor, H. S., B. Temples, and W. V. Shaw. 1972. Staphylococcal delta hemolysin: purification and characterization. Arch. Biochem. Biophys. 151:142–156.
102. Kaplan, M. T. 1972. Specific and nonspecific staining in detecting staphylococcal alpha toxin on erythrocytes by immunofluorescence. Infect. Immun. 5:831–832.
103. Kapral, F. A. 1965. The phagocytosis and intracellular fate of staphylococci. Ann. N.Y. Acad. Sci. 128:285–300.
104. Kapral, F. A. 1966. An analysis of factors involved in an experimental staphylococcal peritonitis and their possible role in other types of staphylococcal infections. Postepy Mikrobiol. 5:297–308.
105. Kapral, F. A. 1967. Neutralization of *Staphylococcus aureus* delta hemolysin by phospholipids. Bacteriol. Proc., p. 64.
106. Kapral, F. A. 1972. Inhibition of *Staphylococcus aureus* delta hemolysin by phospholipids. Proc. Soc. Exp. Biol. Med. 141: 519–521.
107. Kapral, F. A. 1974. *Staphylococcus aureus*: some host parasite interactions. Ann. N.Y. Acad. Sci. 236:267–276.
108. Kapral, F. A., and M. M. Miller. 1971. Product of *Staphylococcus aureus* responsible for the scaled skin syndrome. Infect. Immun. 4:541–545.

109. Kapral, F. A., A. M. Keogh, and J. H. Taubler. 1965. The nature of alpha-toxin production by *Staphylococcus aureus* grown *in vivo*. Proc. Soc. Exp. Biol. Med. 119: 74-77.
110. Kayser, A., and M. Raynaud. 1965. Etude d'une deuxieme hemolysine (distincte de l'hemolysine alpha) presente dans les filtrats de culture de la souche Wood 46 de *Staphylococcus aureus* (hemolysine G ou delta). Ann. Inst. Pasteur Paris 108:215-233.
111. Klainer, A. S., M. A. Madoff, L. Z. Cooper, and L. Weinstein. 1964. Staphylococcal alpha-hemolysin: detection on the erythrocyte membrane by immunofluorescence. Science 145:714-715.
112. Klainer, A. S., T. W. Chang, and L. Weinstein. 1972. Effects of purified staphylococcal alpha toxin on the ultrastructure of human and rabbit erythrocytes. Infect. Immun. 5: 808-813.
113. Klebs, E. 1872. Beitrage zur pathologischen Anatomie der Schusswunde, p. 104-122. Leipzig.
114. Kleck, J. L., and J. H. Donahue. 1968. Production of thermostable hemolysin by cultures of *Staphylococcus epidermidis*. J. Infect. Dis. 181:317-323.
115. Korbecki, M., and J. Jeljaszewicz. 1964. Action of staphylococcal alpha-hemolysin on KB cells. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1: Orig. Reihe B 192:130-433.
116. Korbecki, M., and J. Jeljaszewicz. 1965. Action of staphylococcal toxins in cell cultures. J. Infect. Dis. 115:205-213.
117. Kraus, R., and P. Clairmont. 1900. Uber Hämolysin and Antihämolysine. Wien. Klin. Wochenschr. 13:49-56.
118. Kreger, A. S., and A. W. Bernheimer. 1971. Disruption of bacterial protoplasts and spheroplasts by staphylococcal delta hemolysin. Infect. Immun. 3:603-605.
119. Kreger, A. S., K. S. Kim, F. Zaboretsky, and A. W. Bernheimer. 1971. Purification and properties of staphylococcal delta hemolysin. Infect. Immun. 3:449-465.
120. Kumar, S., and R. K. Lindorfer. 1962. The characterization of staphylococcal toxins. I. The electrophoretic migration of the alpha hemolytic, dermonecrotic, lethal, and leucocidal activities of crude toxin. J. Exp. Med. 115:1095-1106.
121. Kumar, S., K. Locken, A. Kenyon, and R. K. Lindorfer. 1962. The characterization of staphylococcal toxins. II. The isolation and characterization of a homogeneous staphylococcal protein possessing alpha hemolytic, dermonecrotic, lethal and leucocidal activities. J. Exp. Med. 115:1107-1115.
122. Lack, C. H., and D. G. Wailling. 1954. A study of 435 strains of *Staphylococcus pyogenes* with reference to factors which may contribute to pathogenicity. J. Pathol. Bacteriol. 68:431-443.
123. Lack, C. H., and A. G. Towers. 1962. Serologic tests for staphylococcal infection. Br. Med. J. 10 Nov., p. 1227-1231.
124. Leboeuf-Trudeau, T., J. de Repentigny, R. M. Frenette, and S. Sonea. 1967. Inhibitory effects of tryptophan analogues on growth and alpha toxin activity of *Staphylococcus aureus*. Bacteriol. Proc., p. 64.
125. Lominski, I., J. P. Arbuthnott, and J. B. Spence. 1963. Purification of *Staphylococcus* alpha-toxin. J. Pathol. Bacteriol. 86:258-262.
126. Low, D. K. R., J. H. Freer, and J. P. Arbuthnott. 1974. Consequences of sphingomyelin degradation in erythrocyte ghost membranes by staphylococcal beta-toxin (sphingomyelinase C). Toxicon 12:279-285.
127. Madoff, M. A., and L. Weinstein. 1962. Purification of staphylococcal alpha-hemolysin. J. Bacteriol. 83:914-918.
128. Madoff, M. A., M. S. Artenstein, and L. Weinstein. 1963. Studies of the biologic activity of purified staphylococcal alpha-toxin. II. The effect of alpha-toxin on Ehrlich ascites carcinoma cells. Yale J. Biol. Med. 35:382-389.
129. Maheswaran, S. K., and R. K. Lindorfer. 1966. Studies on the mode of action of purified staphylococcal beta hemolysin. Bacteriol. Proc., p. 44.
130. Maheswaran, S. K., and R. K. Lindorfer. 1967. Staphylococcal beta hemolysin. II. Phospholipase C activity of purified beta hemolysin. J. Bacteriol. 94:1313-1319.
131. Maheswaran, S. K., and R. K. Lindorfer. 1970. Purification and partial characterization of staphylococcal delta hemolysin. Bacteriol. Proc., p. 78.
132. Maheswaran, S. K., and R. K. Lindorfer. 1971. Physicochemical and biological properties of purified staphylococcal beta hemolysin. Bacteriol. Proc., p. 80.
133. Maheswaran, S. K., K. L. Smith, and R. K. Lindorfer. 1967. Staphylococcal beta hemolysin. I. Purification of beta hemolysin. J. Bacteriol. 94:300-305.
134. Maheswaran, S. K., S. P. Frommes, and R. K. Lindorfer. 1969. Ultrastructural changes in neutrophils treated with staphylococcal alpha toxin. Can. J. Microbiol. 15:128-129.
135. Maniar, A. C., C. Westlake, and P. Warner. 1967. Prevalence of staphylococcal anti-delta lysin in man. Can. J. Microbiol. 13:925-929.
136. Manohar, M., S. K. Maheswaran, S. P. Frommes, and R. K. Lindorfer. 1967. Platelet damaging factor, a fifth activity of staphylococcal alpha toxin. J. Bacteriol. 94:224-231.
137. Marks, J. 1951. The standardization of staphylococcal alpha antitoxin with special reference to anomalous hemolysins including delta lysin. J. Hyg. (London) 49:52-56.
138. Marks, J. 1952. Recognition of pathogenic staphylococci: with notes on non-specific

staphylococcal haemolysin. J. Pathol. Bacteriol. 64:175–186.
139. Marks, J., and A. C. T. Vaughan. 1950. Staphylococcal delta haemolysin. J. Pathol. Bacteriol. 62:597–615.
140. Marucci, A. A. 1963. Mechanism of action of staphylococcal alpha-hemolysin. II. Analysis of the kinetic curve and inhibition by specific antibody. J. Bacteriol. 86:1189–1195.
141. McLeod, J. W. 1963. Thermostable staphylococcal toxin. J. Pathol. Bacteriol. 86:35–53.
142. McNiven, A. C., and J. P. Arbuthnott. 1971. Cell-associated alpha toxin from *Staphylococcus aureus*. J. Med. Microbiol. 5:123–127.
143. McNiven, A. C., P. Owen, and J. P. Arbuthnott. 1972. Multiple forms of staphylococcal alpha toxin. J. Med. Microbiol. 5:113–122.
144. Meduski, J. W., and P. Hochstein. 1972. Hot-cold hemolysis: the role of positively charged membrane phospholipids. Experientia 15:565–566.
145. Mollby, R., and T. Wadstrom. 1970. Protides of the biological fluids, p. 465–469. Pergamon Press, Oxford.
146. Mollby, R., and T. Wadstrom. 1971. Separation of gamma hemolysin from *Staphylococcus aureus* Smith 5R. Infect. Immun. 3:633–635.
147. Moore, G. L., W. F. Kocholaty, D. A. Cooper, J. L. Gray, and S. L. Robinson. 1970. A proteinase from human erythrocyte membranes. Biochim. Biophys. Acta 212:126–133.
148. Morgan, F. G., and J. J. Graydon. 1936. Toxins of the *Staphylococcus*, with especial reference to the estimation of potency. J. Pathol. Bacteriol. 43:385–401.
149. Morrison, W. L., and H. Neurath. 1953. Proteolytic enzymes of the formed elements of human blood. I. Erythrocytes. J. Biol. Chem. 200:39–51.
150. Mudd, S. 1960. The practicability of enhancing specific resistance to staphylococci infection. J. Am. Med. Assoc. 173:1360–1361.
151. Mudd, S., G. P. Gladstone, and N. A. Lenhart. 1965. The antigenicity in man of staphylococcal leucocidin toxoid, with notes on therapeutic immunization in chronic osteomyelitis. Br. J. Exp. Pathol. 46:455–472.
152. Murphy, R. A., and R. Haque. 1967. Purification and properties of staphylococcal delta hemolysin. I. Production of delta hemolysin. J. Bacteriol. 94:1327–1333.
153. Nelis, P. 1935. Contribution a l'etude de la toxine staphylococcique et de ses derives (antitoxine et anatoxine staphylococcique). Bull. Acad. Med. Belg. 15:539–568.
154. Noble, W. C. 1966. Virulence and the biochemical characters of staphylococci. J. Pathol. Bacteriol. 91:181–193.
155. Nogrady, G., and A. L. Burton. 1961. L'effect de la toxine staphylococcique alpha sur les cellules d'embryon de poulet en cultures de tissus. Pathol. Biol. 9:831–833.
156. Noll, H. 1966. Chain initiation and control of protein synthesis. Science 151:1241–1245.
157. Nosal, R., H. Raskova, and K. Masek. 1969. Comparative study of the effect of staphylococcal alpha-toxin on erythrocytes and thrombocytes. Toxicon 7:255–262.
158. Novak, E., J. Seifert, and H. Raskova. 1970. Effect of staphylococcal alpha toxin on mitochondrial Mg^{2+} (Na^+, K^+) activated adenosine triphosphatase. Toxicon 8:261–270.
159. Novak, E., J. Seifert, E. Buchar, and H. Raskova. 1971. Effect of staphylococcal alpha toxin on the phosphorylation of ADP by rat liver mitochondria-I. Inhibition of phosphorylation by a thermo-stable factor. Toxicon 9:361–366.
160. Novak, E., J. Seifert, E. Buchar, and H. Raskova. 1971. Effect of staphylococcal alpha toxin on the phosphorylation of ADP by rat liver mitochondria-II. Effect of staphylococcal alpha toxin upon electron transport. Toxicon 9:361–366.
161. Paradisi, F., and P. D'Aniello. 1969. Effect of serum albumin on the damage induced by *Staphylococcus* alpha-toxin in cell cultures. G. Microbiol. 17:101–106.
162. Parker, J. T., J. G. Hopkins, and A. Gunther. 1926. Further studies on the production of *Staphylococcus aureus* toxin. Proc. Soc. Exp. Biol. Med. 23:344–346.
163. Rahal, J. J. 1972. Comparative effects of purified staphylococcal alpha and delta toxins on mitochondrial metabolism. J. Infect. Dis. 126:96–103.
164. Rahal, J. J., M. E. Plaut, H. Rosen, and L. Weinstein. 1967. Purified staphylococcal alpha toxin: effect on epithelial ion transport. Science 155:1118–1120.
165. Remsen, C. C., S. W. Watson, and A. W. Bernheimer. 1970. Evidence for an ordered arrangement in erythrocyte membranes. Biochem. Biophys. Res. Commun. 40:1297–1304.
166. Richou, R. 1953. Recherches sur l'immunite antistaphylococcique beta naturellement acquise chez l'homme et chez differentes especes animals. Bull. Acad. Nat. Med. Paris 137:446–450.
167. Robinson, J., and F. S. Thatcher. 1963. Studies with staphylococcal toxins. VII. Separation of a proteolytic enzyme from alpha hemolysin. Can J. Microbiol. 9:697–702.
168. Robinson, J., F. S. Thatcher, and J. Gagnon. 1958. Studies with staphylococcal toxins. IV. The purification and metallic requirements of specific hemolysins. Can. J. Microbiol. 4: 345–361.
169. Rogers, D. E., and M. A. Melly. 1965. Speculations on the immunology of staphylococcal infections. Ann. N.Y. Acad. Sci. 128:274–284.
170. Samanek, M., and F. Zajic. 1965. Proceedings of the second international conference on pharmacology management, Prague, 1963, vol. 9, p. 199. Pergamon Press, Oxford.
171. Schwabacher, H., A. C. Cunliffe, R. E. O. Williams, and G. J. Harper. 1945. Hyal-

uronidase production by staphylococci. Br. J. Exp. Pathol. 26:124-129.

172. Seeman, P. 1974. Ultrastructure of membrane lesions in immune lysis, osmotic lysis and drug-induced lysis. Fed. Proc. 33:2116-2124.
173. Sharma, B. S., and R. Haque. 1973. Chemically-defined medium for production of staphylococcal beta-lysin. Can. J. Microbiol. 19: 1319-1323.
174. Sharma, B. S., and R. Haque. 1973. Effect of tryptophan analogues on synthesis of staphylococcal beta-haemolysin. J. Gen. Microbiol. 77:221-224.
175. Six, H. R., and S. Harshman. 1973. Purification and properties of two forms of staphylococcal alpha toxin. Biochemistry 12:2672-2676.
176. Six, H. R., and S. Harshman. 1973. Physical and chemical studies on staphylococcal alpha toxins A and B. Biochemistry 12:2677-2682.
177. Slanetz, L. W., and C. H. Bartley. 1953. The diagnosis of staphylococcal mastitis, with special reference to the characteristics of mastitis staphylococci. J. Infect. Dis. 92: 139-151.
178. Smith, M. L., and S. A. Price. 1938. *Staphylococcus* gamma haemolysin. J. Pathol. Bacteriol. 47:379-393.
179. Smith, I. N., A. P. Wilson, E. C. Hazard, W. K. Hummel, and M. E. Dewey. 1960. Death from staphylococci in mice. J. Infect. Dis. 107:369-378.
180. Sonea, S., J. de Repentigny, and A. Frappier. 1965. A. reversible metabolic control of virulence characteristics of a toxinogenic *Staphylococcus aureus* strain. Can. J. Microbiol. 11:383-394.
181. Součková, A., and A. Soucék. 1972. Inhibition of the hemolytic action of alpha and beta lysins of *Staphylococcus pyogenes* by *Corynebacterium hemolyticum*, *C. ovis*, and *C. ulcerans*. Toxicon 10:501-509.
182. Speth, V., D. F. H. Wallach, E. Weidekamm, and H. Knüfermann. 1972. Micromorphologic consequences following perturbation of erythrocyte membranes by trypsin, phospholipase A, lysolecithin, sodium dodecyl sulphate and saponin: a correlated freeze-etching study. Biochim. Biophys. Acta 255:386-394.
183. Symington, D. A., and J. P. Arbuthnott. 1969. Effects of *Staphylococcus* alpha-toxin and streptolysin S on the oxidation of succinate by ascites tumour cells. J. Med. Microbiol. 2:495-505.
184. Taubler, J. H., F. A. Kapral, and S. Mudd. 1963. Role of alpha-toxin in lesion formation by *Staphylococcus aureus* on sutures subcutaneously implanted in mice. J. Bacteriol. 86:51-57.
185. Taylor, A. G., and M. Plommet. 1973. Anti-gamma haemolysin as a diagnostic test in staphylococcal osteomyelitis. J. Clin. Pathol. 26:409-412.
186. Taylor, A. G., and A. W. Bernheimer. 1974 Further characterization of staphylococcal gamma hemolysin. Infect. Immun. 10:54-59.
187. Thal, A., and W. Egner. 1954. Local effect of staphylococcal toxin. Studies on blood vessels with particular reference to phenomenon of dermonecrosis. Arch. Pathol. 57:392-404.
188. Thal, A., and W. Egner. 1961. Site of action of *Staphylococcus* alpha toxin. J. Exp. Med. 113:67-82.
189. Thelestam, M., R. Mollby, and T. Wadstrom. 1973. Effects of staphylococcal alpha-, beta-, delta-, and gamma-hemolysins on human diploid fibroblasts and HeLa cells: evaluation of a new quantitative assay for measuring cell damage. Infect. Immun. 8:938-946.
190. Turgeon, P., S. Grimard, and J. de Repentigny. 1966. Inhibitions produites par la 8-azaguanine chez une souche toxinogène de *Staphylococcus aureus*. Can. J. Microbiol. 12:588-590.
191. Uchiyama, C., M. Onizuka, T. Takeuchi, and M. Nagamatsu. 1969. Effect of bacteria and bacterial toxin on tissue culture cells. Report 2. Effect of staphylococcal toxin and anti-toxin on the cultured rabbit kidney cells. J. Kurume Med. Assoc. 32:1584-1586.
192. Uyeda, C. T., B. Gerstl, J. K. Smith, and W. T. Carr. 1966. Antistaphylococcal beta-hemolysin antibodies in humans with neurological disease. Proc. Soc. Exp. Biol. Med. 123: 143-146.
193. Van de Velde, H. 1894. Etude sur le mecanisme de la virulence du *Staphylocoque pyogene*. Cellule 10:401-460.
194. Vogt, W. 1970. What is a toxin? Toxicon 8: 251.
195. Wadstrom, T. 1968. Studies on extracellular proteins from *Staphylococcus aureus*. IV. Separation of alpha toxin by isoelectric focusing. Biochim. Biophys. Acta 168:228-242.
196. Wadstrom, T., and R. Mollby. 1971. Studies on extracellular proteins from *Staphylococcus aureus*. VI. Production and purification of beta hemolysin in large scale. Biochim. Biophys. Acta 242:288-307.
197. Wadstrom, T., and R. Mollby. 1971. Studies on extracellular proteins from *Staphylococcus aureus*. VII. Studies on beta hemolysin. Biochim. Biophys. Acta 200:308-320.
198. Wadstrom, T., and R. Mollby. 1972. Some biological properties of purified staphylococcal hemolysins. Toxicon 10:511-519.
199. Wadstrom. T., M. Thelestam, and R. Mollby. 1974. Biological properties of extracellular proteins from *Staphylococcus*. Ann. N.Y. Acad. Sci. 236:343-361.
200. Walbum, L. E. 1921. L'action de divers sels metaliques sur la production de staphylolysine. C. R. Soc. Biol. 85:376-377.
201. Walbum, L. E. 1921. Action de la staphylolysine sur les globules de chevre. C. R. Soc. Biol. 85:1205-1206.
202. Walbum, L. E. 1922. Studien über die Bildung der bakteriellen Toxine.-I. Mitteilung.

Staphylolysin. Biochem. Z. 129:367–443.
203. Watanabe, M., and I. Kato. 1974. Purification and properties of staphylococcal alpha-toxin. Jpn. J. Med. Sci. Biol. 27:86–89.
204. Weissman, G., G. Sessa, and A. W. Bernheimer. 1966. Staphylococcal alpha toxin: effects on artificial lipid spherules. Science 154:772–774.
205. Wiegershausen, B. 1960. Uber einige pharmakologische Eigenschaften von Staphylolysin. Arch. Exp. Pathol. Pharmacol. 238: 144–145.
206. Wiegershausen, B. 1962. On the pharmacology of staphylococcal toxin (Wood 46). V. The effect of *Staphylococcus* toxin on the isolated heart, auricles and vessels of various species. Acta Biol. Med. Ger. 9:517.
207. Williams, R. E. O., and G. J. Harper. 1947. Staphylococcal haemolysins on sheep-blood agar with evidence for a fourth haemolysin. J. Pathol. Bacteriol. 59:69–78.
208. Wiseman, G. M. 1965. Some characteristics of the beta haemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. 89:187–207.
209. Wiseman, G. M. 1965. Factors affecting the sensitization of sheep erythrocytes to staphylococcal beta lysin. Can. J. Microbiol. 11: 463–471.
210. Wiseman, G. M. 1968. The nature of staphylococcal beta hemolysin. II. Effect on mammalian cells. Can. J. Microbiol. 14:179–181.
211. Wiseman, G. M. 1970. *In* S. J. Ajl et al. (ed.), Microbial toxins, a comprehensive treatise, vol. 3, chapt. 6. Academic Press Inc., New York.
212. Wiseman, G. M., and J. D. Caird. 1966. Mode of action of the beta hemolysin of *Staphylococcus aureus*. Can. Soc. Microbiol. Abstr., p. 16.
213. Wiseman, G. M., and J. D. Caird. 1967. The nature of staphylococcal beta hemolysin. I. Mode of action. Can. J. Microbiol. 13:369–376.
214. Wiseman, G. M., and J. D. Caird. 1968. Phospholipase activity of the delta hemolysin of *Staphylococcus aureus*. Proc. Soc. Exp. Biol. Med. 128:428–430.
215. Wiseman, G. M., and J. D. Caird. 1970. Mode of action of the alpha toxin of *Staphylococcus aureus*. Can. J. Microbiol. 16:47–50.
216. Wiseman, G. M., and J. D. Caird. 1972. Further observations on the mode of action of the alpha toxin of *Staphylococcus aureus* "Wood-46". Can. J. Microbiol. 18:987–992.
217. Wiseman, G. M., J. D. Caird, and H. B. Fackrell. 1975. Trypsin-mediated activation of the alpha haemolysin of *Staphylococcus aureus* "Wood-46". J. Med. Microbiol. 8:29–38.
218. Wurzel, M., A. W. Bernheimer, and B. W. Zweifach. 1966. Staphylococcal alpha toxin: induced contraction of isolated arterial smooth muscle. Am. J. Physiol. 210:360–364.
219. Yoshida, A. 1963. Staphylococcal delta hemolysin. I. Purification and chemical properties. Biochim. Biophys. Acta 71:544–553.